An evaluation of the *Nippostrongylus brasiliensis* infection in the rat as a model for the development of subunit vaccines against nematode parasites of ruminants

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Contents

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Abstract

Nematode parasites of the gastro-intestinal (GI) tract, including species of the genus *Trichostrongylus,* are a major cause of pathology and loss of production in sheep and goat farming world wide. Such infections are currently controlled by prophylactic drenching with several classes of anthelmintic drugs. Spreading drug resistance and public concern over drug residues in food have led to research into vaccination as an alternative means of controlling these parasite species.

Nippostrongylus brasiliensis is a GI nematode parasite of rodents and is an extensively applied laboratory model for defining the immune mechanisms that mediate worm expulsion. *N. brasiliensis* infection in the rat shares many similarities with *Trichostrongylus* infection in ruminants including, importantly, the functional proteins excreted or secreted by the nematode (ES proteins). Several of these ES proteins are potential candidates for vaccines. The aim of this project was to use the rat / *Nippostrongylus* parasite system as a model for vaccination with recombinant ES proteins.

Prior to vaccination trials *N. brasiliensis* infections of varying levels were investigated and immunological assays were developed to allow the assessment of immune responses to infection and vaccination. The assays measured specific antibody and Mast Cell Protease (RMCP II) by ELISA. The utility of measuring immune cytokines by RT PCR was also investigated.

Two proteins of interest, a superoxide dismutase (SOD) and acetylcholinesterase (AChE), were selected as vaccine candidates. Recombinant AChE was kindly provided for study by Prof Murray Selkirk. A cDNA encoding the SOD was amplified by PCR with a functional enzyme being obtained after expression in *Escherichia coli.* Sequence analysis of the predicted recombinant protein sequence is described along with activity and stage specificity of expression of the native protein

Vaccination with these two recombinant enzymes was investigated to ascertain their protective capacity and thus their suitability as vaccine candidates. In a preliminary trial with AChE, vaccinated animals showed a 48% reduction in egg output compared to controls, this being associated with changes in antibody and

RMCP II levels. The SOD did not induce any protection or a significant immune response. Further trials investigated how the route of administration and adjuvant might affect the protective capacity of these proteins, with protection varying between 0 and 38%. The relevance of these results to future vaccine trials in ruminants is discussed.

Abbreviations

aa	amino acids
AChE	acetylcholinesterase
ANOVA	analysis of variance
APS	ammonium persulphate
bp	base pairs
°C	degrees centigrade
cDNA	complementary deoxyribonucleic acid
cm	centimetre
dH_2O	distilled water
DNA	deoxyribonucleic acid
DPI	days post infection
DNTB	dithiobis-2-nitrobenzoic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ES	excreted or secreted products
EST	expressed sequence tag
EtBr	ethidium bromide
FEC	faecal egg count
g	gram
g	gravitational force
GL	globule leucocyte
GABA	gamma-aminobutyric acid
GI	gastro-intestinal
h	hour
HRP	horse radish peroxidase
Ig	immunoglobulin
IL	interleukin
IN	intra-nasal
IP	intra-peritoneal

IPTG	isopropyl-1-thio-β-D-galactopyranoside
kb	kilobases
kDa	kilo Dalton
kg	kilogram
L	litre
L3 larvae	(infective) third larval stage
m	metres
M	Molar
mg	milligram
min	minutes
ml	millilitre
mM	millimolar
mm	millimetre
mRNA	messenger ribonucleic acid
μg	microgram
μl	microlitre
μM	micromolar
ng	nanogram
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pers. comm.	personal communication
pH	$-\log_{10}$ (hydrogen ion concentration)
rAChE	recombinant acetylcholinesterase
RMCP II	rat mast cell protease II
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcriptase
RT PCR	reverse transcription polymerase chain reaction
s	second
S1	water soluble protein extract

SC	sub-cutaneous
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
sp.	species
TAE	tris acetic acid EDTA buffer
TBS	tris buffered saline
TBST	tris buffered saline with Tween-20
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)amino methane
Triton x-100	octylphenoxypolyethoxyethanol
Tween-20	sorbitan monolaurate
U	unit
UV	ultraviolet
V	volt
v/v	volume per volume
w	week
W	watt
w/v	weight per volume

Chapter 1 – Introduction

1.1 Introduction

Gastro-intestinal (GI) nematode parasite species are a major problem for livestock production, both in the UK and the rest of the world. They cause gastro-intestinal disease referred to as parasitic gastro-enteritis. The common symptoms in ruminants are of inflammation, inappetance and scouring (diarrhoea). These symptoms lead to malabsorption of nutrients from the gut with resultant poor weight gain and growth in infected animals and malnutrition that may ultimately lead to death in heavily infected animals.

Substantial economic losses result directly from the reduced productivity effects on livestock and a cost is also incurred from the measures needed to control infection. This latter cost alone was estimated at £1000 million world-wide in 1994 based on anthhelmintic sales figures (Newton and Munn, 1999).

The currently used control methods for ruminant GI nematodes are chemical control and pasture management. Frequent dosing with anthelmintic drugs is the primary means of control. The development of resistance to anthelmintic drugs in GI nematode populations has led to an increased interest in the development of vaccines against the parasites.

Intestinal nematode species of importance in sheep in the UK are two species of the genus *Trichostrongylus* inhabiting the small intestine, *Trichostrongylus vitrinus* and *Trichostrongylus colubriformis* (Reid and Armour, 1975; Coop *et al.*, 1979). A laboratory model of nematode infection of the small intestine may be useful in the development of vaccines against *Trichostrongylus sp.* allowing antigen candidates, delivery systems, adjuvants and challenge regimes to be tested in small scale trials.

Nippostrongylus brasiliensis is a rodent intestinal nematode that has commonly been used as a laboratory model of GI nematode infection (reviewed; Ogilvie and Jones, 1971). Although the third larval (L3) stage follows a tissue migratory phase of infection, the L4 and adult stages inhabit the small intestine and induce similar pathology and immune responses to *Trichostrongylus sp.* These

similarities in the niche of the adult worms and in the pathology and immune responses to both species are the basis of the infection model.

1.2 Phylogeny of nematode parasites

Nematode worms form the distinct phylum Nematoda. *Trichostrongylus sp.* and *N. brasiliensis* belong to the order Strongylida, which contains 4 sub-orders and seven superfamilies of animal parasitic nematodes (Blaxter *et al.*, 1998; Durette-Desset *et al.*, 1994)

Trichostrongylus sp. are members of the superfamily Trichostrongyloidea and the family Trichostrongylidae (Chilton *et al.*, 2001; Durette-Desset *et al.*, 1999). This group contains several other important parasites of animals and in particular of ruminants, including *Haemonchus contortus, Ostertagia ostertagi*, and *Teladorsagia circumcincta.*

N. brasiliensis belongs to the closely related superfamily Heligmosomatoidea. This taxum is grouped with the superfamily Trichostrongyloidea of ruminant parasites in the sub-order Trichostrongylina (Durette-Desset *et al.*, 1994).

The molecular phylogenetic tree of the Nematoda constructed using small subunit ribosomal DNA sequences groups *N. brasiliensis* very closely with *H. contortus* and other Trichostrongylid nematodes in the order Strongylida (Blaxter *et al.*, 1998). This indicates a high degree of similarity between the species at a molecular genetic level.

1.3 Life cycle of nematode parasites

1.3.1 Trichostrongylidae

Trichostrongylus sp. have a direct lifecycle, typical of the Trichostrongylidae and illustrated in Figure 1.1. Eggs deposited on pasture in the faeces and hatch and develop to L3 stage larvae in around 1-2 weeks in optimal environmental conditions. The L3 larvae are ingested directly by the grazing host animal from the pasture and pass through the digestive tract exsheathing in the abomasum. On entering the small intestine the L3 penetrate and burrow into the mucosa. Here the worms go through their remaining moults to immature adult (L5) at around 12 days and to mature adult at around 16 days after infection. Eggs are passed in the faeces by around 2-3 weeks following infection (Urquhart *et al.*, 1987).

1.3.2 *Nippostrongylus brasiliensis*

N. brasiliensis life cycle, shown in Figure 1.2, is also direct although the larvae infect the host through the skin and migrate to the intestine via the lungs. Eggs are passed in the faeces and hatch with the larvae developing to infective L3 stage in around a week. When the larvae contact the skin of the host they rapidly penetrate the dermal tissues (Lee, 1972). They then migrate through the venous circulation, penetrating the alveolar spaces of the lungs around 24-72 h following infection where they moult to L4 stage (Croll, 1977; Tindall and Wilson, 1990). The L4 are passed up the trachea and swallowed. Larvae migrate to a site in the anterior small intestine and go through the remaining moults to emerge as sexually mature adults on the surface of the mucosa after around 5 days (Alphey, 1970).

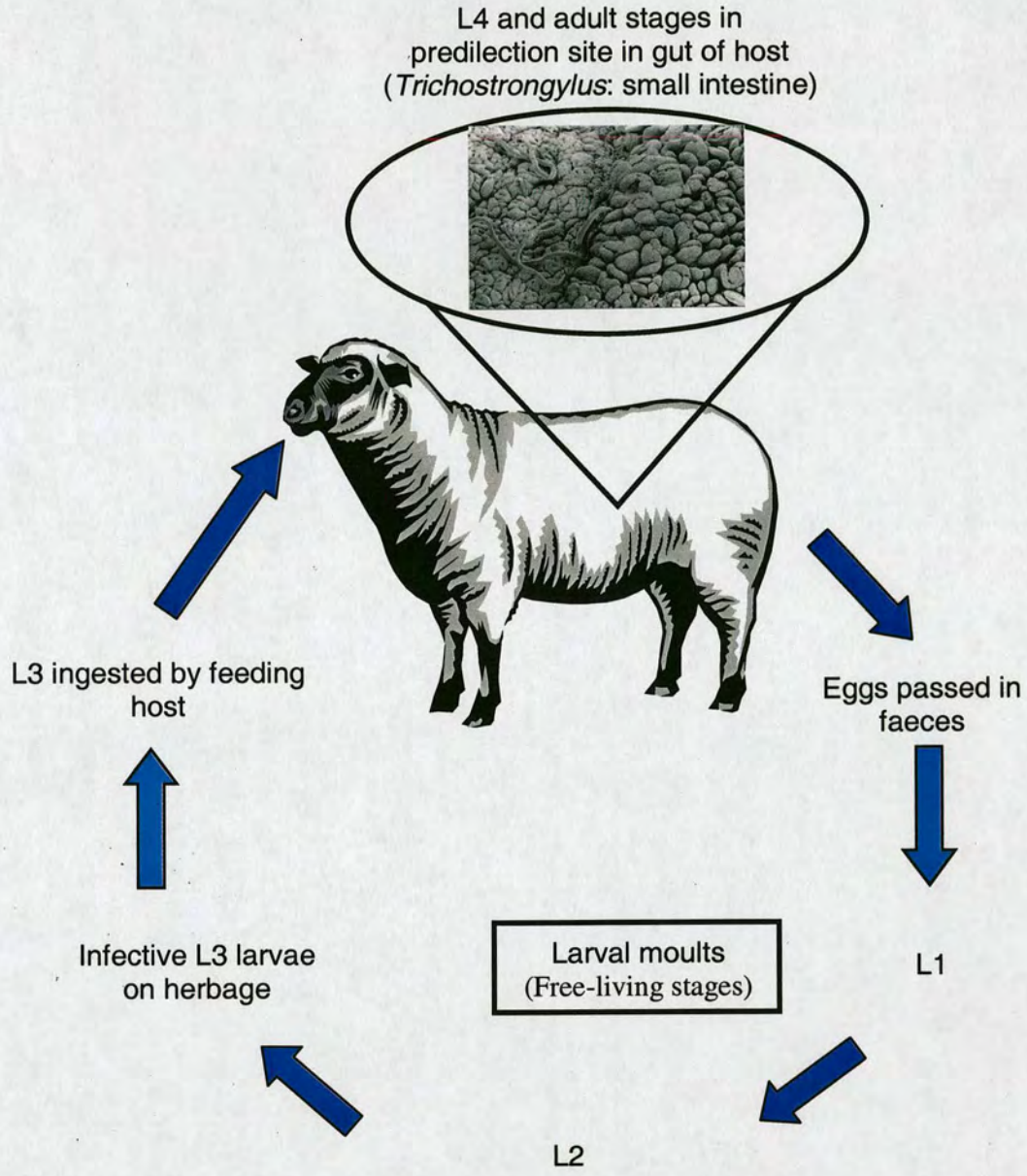


Figure 1.1. The life cycle of Trichostrongyloid nematodes parasitic in the sheep gastro-intestinal tract. Eggs passed in the faeces of the host sheep hatch to free living larval stages. The infective L3 stage is ingested by the feeding sheep and the L4 and adult stages mature in the predilection site, in the case of the illustrated example, *Trichostrongylus vitrinus,* the small intestine (Photograph, Moredun Research Institute).

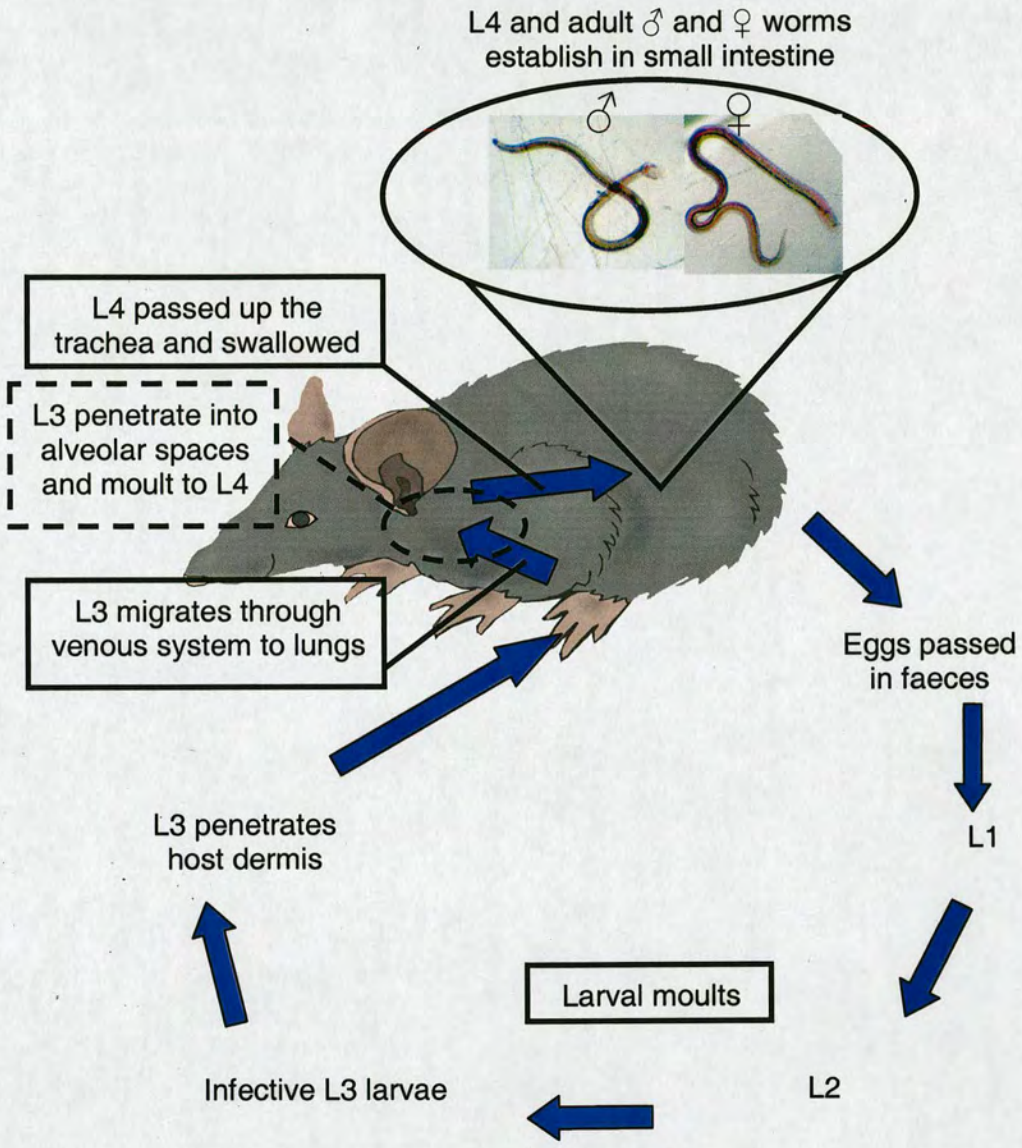


Figure 1.2. The life cycle of *Nippostrongylus brasiliensis* in the rat host Eggs passed in the faeces hatch and larvae moult through L1 and L2 free-living forms. The L3 infective larvae enter the host by skin penetration and go through a tissue migratory phase passing through the veinous system and entering the lung alveoli. Larvae are then passed up the airways and swallowed. L4 and adult worms then enter the small intestinal phase of the life cycle. (Photograph, Moredun Research Institute, G Ball).

1.4 Epidemiology of GI nematode infection

1.4.1 *Trichostrongylus* infection in sheep

Sheep acquire nematode infections from infected pasture as they graze. Infections usually occur as mixed infections of several species with *T. vitrinus* in the small intestine and *T. circumcincta* in the abomasum as the species of most prevalence in the UK. *T. colubriformis* is the more common intestinal species in other temperate regions such as Australia (Reid and Armour, 1975; Coop *et al.*, 1979).

Infection rate is linked to the rate of ingestion of infective larvae, which is dependent on how heavily the pasture is infected, and on the susceptibility of host animals. Infection rate in the UK is typically high during summer and autumn, dropping to lower levels over the winter as exposed ewes and lambs acquire immunity to infection and pasture contamination decreases (Urquhart *et al.*, 1987).

Larvae of *Trichostrongylus sp.* have a high capacity to survive cold or dry conditions and many larvae will over winter and remain viable to infect animals in the spring. In some areas climatic conditions can allow *Trichostrongylus sp.* larvae to survive in sufficient numbers to produce severe problems in the spring (Urquhart *et al.*, 1987). In the UK, however, numbers of over wintering *T. vitrinus* larvae are lower and mortality among the larval population is high during the winter. Warmer spring and summer temperatures decrease the development time and mortality rate of larvae on pasture if there is adequate humidity. Such conditions allow increased rates of infection during this time (Rose and Small, 1984).

Numbers of *Trichostrongylus spp* in grazing lambs in the UK, are usually low during spring and early summer indicating a low infection rate from over wintering larvae (Cornwell, 1975). The phenomenon of periparturient relaxation of acquired immunity, whereby previously exposed and immune ewes lose their immunity around the time of parturition, leads to an increase in egg output in summer, exposing new-born lambs to high infection rates. Worm numbers in lambs accumulate in late summer and early autumn, at which time *Trichostrongylus spp* (predominantly *T. vitrinus*) along with *T. circumcincta* become the most common

nematode parasites (Cornwell, 1975). Clinical effects of parasitic gastro-enteritis associated with these species tend to occur at this time.

1.4.2 Laboratory infection regimes with *Nippostrongylus brasiliensis*

Single challenge infections on naïve rats with between two to three thousand, *N. brasiliensis* L3 larvae have a characteristic 10-14 day infection cycle. The faecal egg count (FEC), and also worm numbers, peak at around seven days post infection. From day 7 onwards worm numbers decrease, with the infection clearing completely by day 10-14. This has been the most commonly used and described laboratory infection regime with *N. brasiliensis* (Ogilvie and Jones, 1971; Rothwell, 1989).

An infection regime using a trickle infection, whereby the rats are continually infected with a low numbers of larvae (five per day for five days a week) has been shown to have a different profile of infection (Jenkins and Phillipson, 1971). In this case the worm burden can increase steadily through the infection period and a sustained chronic infection can occur with worm numbers being maintained even after infection stops as seen in Figure 1.3 (Jenkins and Phillipson, 1971). This situation is much closer to naturally occurring nematode infections and is similar to the pattern in ruminant, parasitic nematode infections acquired from contaminated pasture.

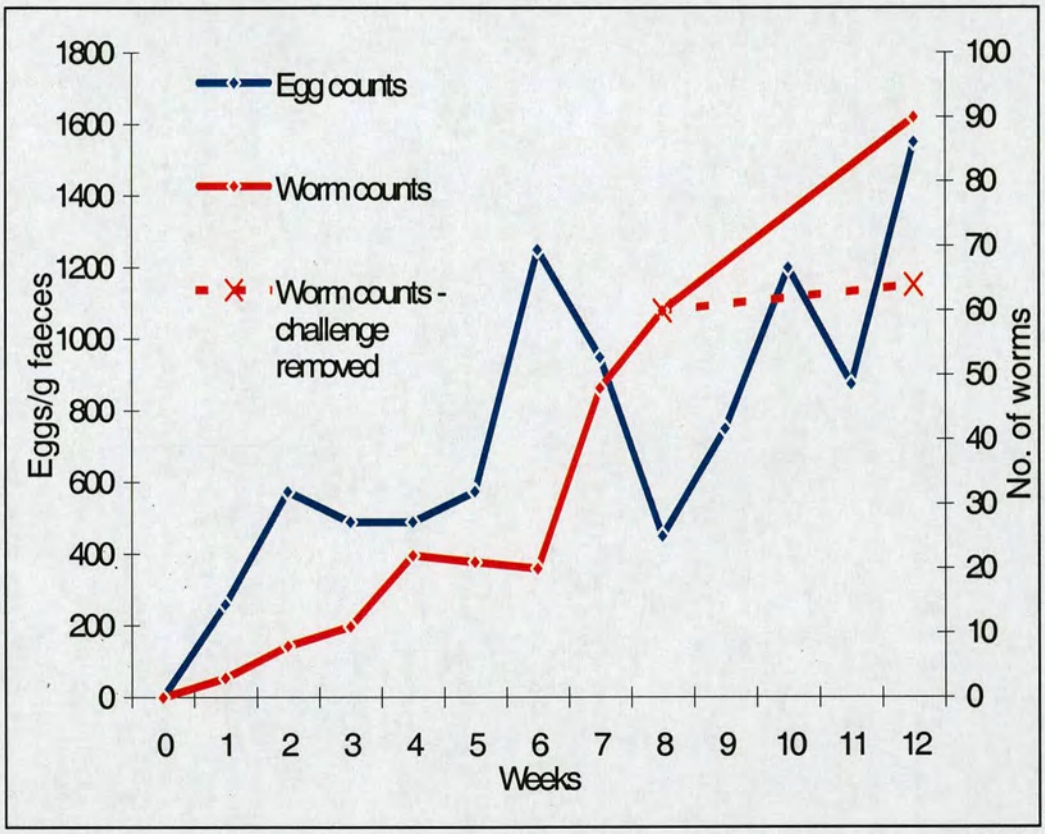


Figure 1.3. The accumulating mean faecal egg counts and adult worm numbers of a group of 36 rats infected via a trickle challenge regime with 5 *Nippostrongylus brasiliensis* L3 per day over the course of 12 weeks. The dashed line indicates the number of worms persisting in a group of animals which were removed from the infection regime after 8 weeks of the experiment (adapted from Jenkins and Phillipson, 1971).

1.5 Parasitic Gastro Enteritis and the pathology of infection

Parasitic gastro-enteritis is the generic term used to describe the clinical symptoms produced by infection with parasitic GI nematodes. Most field infections comprise concomitant infections with more than one species of nematode (Reid and Armour, 1975; Bartley *et al.*, 2003). General symptoms include inappettance, weight loss, poor fleece and carcass quality at slaughter and scouring. These symptoms result from the damage induced by the infection and even in sub-clinical infections pathology can be significant and result in economic losses (Urquhart *et al.*, 1987).

1.5.1 Pathology during *Trichostrongylus* infections

Pathology is similar in both *T. vitrinus* (Taylor and Pearson, 1979) and *T. colubriformis* (Barker, 1975a) infections, although it may be more marked in the latter. The major harmful effect is damage to the mucosa of the small intestine with resultant intestinal inflammation and reduced nutrient uptake. Heavy infections may also induce scouring, leading to further serious effects on the health of the animal (Sykes and Coop, 1976).

Effects are most evident in the proximal 3-4 m of the small intestine where the worms are present in sub-epithelial tunnels (Barker, 1975a; 1975b). In this region there is marked villous atrophy, or flattening of the intestinal villi, the crypts are elongated and the epithelial cells are flattened with unusual morphology (Coop *et al.*, 1979). Some breaks in the epithelial surface occur that may be due to rupture of the worm tunnels. Inflammation of the mucosa is associated with the eroded epithelium (Taylor and Pearson, 1979). Barker (1975b) also demonstrated an increase in the vascular permeability of the infected region, which may lead to loss of plasma and protein into the gut.

More distal regions show reduced villous atrophy and after around 5-7 months after infection show normal morphology and histology. In one study on *T. colubriformis* an increase in villous length in the distal portion of the small

intestine was seen after day 15. This was suggested to be an adaptive response to the reduced surface area and nutrient uptake of the damaged region (Roy *et al.*, 1996).

The severity of pathology also decreases with lower infection dose or rate and pathology becomes less severe as the infection develops (Taylor and Pearson, 1979; Coop *et al.*, 1979). Villous atrophy becomes subtotal regressing proximally and becomes focal, forming characteristic "fingerprint" lesions in areas occupied by the worms. This coincides with the onset of host resistance to incoming larvae, and may occur earlier in *T. vitrinus* than *T. colubriformis* (Jackson *et al.*, 1983).

Changes in activity of brush border associated and other mucosal enzymes has been reported during infection (Jones, 1982). Reduction in the serum level of one of these enzymes, alkaline phosphatase, similar to the change in mucosal level, has been associated with reduction in bone density and size during a mixed *T. vitrinus* / *T. circumcincta* infection (Thamsborg and Hauge, 2001).

As a result of pathological changes in the gut, protein deposition and skeletal mineral deposition are reduced in infected lambs compared to uninfected controls leading to the reduced growth and weight gain effects seen in the former (Sykes *et al.*, 1979). These effects probably result from the reduced uptake of nutrients across the damaged mucosa. Loss of plasma proteins leaking into the gut may further reduce the protein deposition along with the increased protein turnover required to repair the worm induced damage to the intestinal mucosa (Sykes and Coop, 1976; Sykes *et al.*, 1979).

1.5.2 Pathology during *Nippostrongylus* infection

N. brasiliensis infection results in similar pathological changes in the rat intestine. Here pathology is also confined largely to the proximal end of the small intestine or jejunum. Thickening of the mucosa and inflammation of the villi and crypts of the jejunum result from the infection (Simaren and Ogunyoye, 1973). The villi of *Nippostrongylus* infected rats become shortened and irregular compared to those in uninfected rats or in distal, uninfected areas of the same host. The microvilli on the cell surfaces also become shortened and irregular (Symons, 1976). Levels of

activity of brush border enzymes are reduced in a manner similar to those seen in *Trichostrongylus* infections (Symons and Fairbairn, 1963).

The villous atrophy during *N. brasiliensis* infection has been associated with increased apoptosis of epithelial cells on the villous surface (Hyoh *et al.*, 1999). This increased epithelial cell turnover may lead to undifferentiated crypt cells being present on the villi surface explaining some of the altered morphology seen (Symons, 1965).

The villous atrophy produced during *Nippostrongylus* infection, although often described by authors as very similar, is generally somewhat less severe than in *Trichostrongylus* infections of sheep. One possible reason is that the adult worms do not burrow through the mucosal epithelium, but are present coiled around or between villi (Symons, 1976).

Intestinal malabsorption of nutrients including glucose and amino acids also occurs during *Nippostrongylus* infection in rats. This has been explained by the changes to the morphology of villi and cells in the jejunum and to the reduction in the levels of activity of mucosal enzymes such as maltase aminopeptidase and alkaline phosphatase in this area (Symons *et al.*, 1971). Reductions in serum protein and glucose levels and reduced weight gain in heavy infections are likely to result from this malabsorption and inappetance induced by worm damage (Simaren and Ogunyoye, 1973).

Significant pathology in *N. brasiliensis* infection also results from the migrating larvae penetrating the alveolar walls of the lungs. This is characterised by focal haemorrhage accompanied by accumulation of inflammatory cells at the site of larval penetration (Ramaswamy *et al.*, 1991). Infected lungs may be spotted or mottled with haemorrhages for several days after infection. Pathological changes in the lung are more marked during repeat infections when cell accumulation and granuloma formation around incoming larvae are enhanced and persist for much longer (Salman and Brown, 1980; Ramaswamy *et al.*, 1991).

1.6 Resistance and expulsion of gastro-intestinal nematode infection

Adult sheep exposed to repeated nematode challenges acquire an effective immunity to infection over time and will eventually expel a primary worm burden. Following this, they have immunity to further repeated challenges (Balic *et al.*, 2000). A similar immunity develops in repeatedly challenged laboratory rodent models (reviewed; Miller, 1984; Rothwell, 1989). This response varies with the infection level and there are also marked differences in the ability of animals to respond to challenge. This varies between individuals, with age and with reproductive status.

The development of resistance and expulsion of both ruminant and rodent nematodes follow similar patterns, and similar effector processes may be involved. The events surrounding expulsion and the development of resistance are outlined below and a more detailed outline of the immune responses that underlie these events is discussed in the next section (1.7 Immune Responses to Infection).

1.6.1 Resistance and expulsion in *Trichostrongylus* infection of sheep

Adult animals exposed to repeated *Trichostrongylus* infection acquire an effective immunity to infection over time. Continuous natural or experimental trickle infections with differing levels of infective *Trichostrongylus* larvae give cumulative worm burdens up to a threshold level of infection. Once this level has been exceeded establishment of incoming worms is prevented and the established worms are expelled by the immune response. The time taken to reach this threshold level is dependent on the rate of infection. Total worm burden can peak at 20-30,000 prior to expulsion at a high rate of infection whereas a cumulative worm burden at a low infection rate can reach 1,500 without eliciting expulsion (Waller and Thomas, 1981). Following expulsion of a primary infection, animals have immunity to reinfection with further challenges.

Experimental challenges and studies of natural infections have shown that the development of an immune response and the onset of expulsion during a natural or continuous trickle infection follow a distinct pattern. This is characterised first by the resistance to establishment of incoming infective larvae followed by expulsion of established L4 and adult worms (Waller and Thomas, 1981). The exclusion of incoming larvae in this manner followed by expulsion of adults is similar to the sequence of events observed during infection with various nematode species in rodent hosts (Rothwell, 1989). Immune exclusion of incoming larvae may be associated with trapping in mucus while the expulsion of established adult worms is associated with a range of developing humoral and cellular responses (Miller, 1984).

Young animals are susceptible to infection and are not protected or able to expel worms until they reach a critical age. Lambs are unable to mount a protective immune response up to 36 weeks of age (Gibson and Parfitt, 1972; Dobson *et al.*, 1990). The reasons behind this lack of response have not been defined but are believed to involve the immaturity of the immune response. In one study the authors reported that the lack of protection may be related to a reduced globule leukocyte response seen in young lambs (<6 months of age) compared with older counterparts (Gregg *et al.*, 1978).

Protective immunity is relaxed in periparturient and lactating ewes previously immune to infection, leading to an increase in infection rate and a rise in egg output onto pasture during spring (O'Sullivan and Donald, 1973). The mechanism behind this effect is not fully understood but may involve protein resource needs at this time (Coop and Kyriazakis, 1999). This feature of ruminant nematode infections is very important in their epidemiology, being a major source of infection for weaning lambs (Cornwell, 1975; Urquhart *et al.*, 1987).

1.6.2 Resistance and expulsion of *Nippostrongylus* infection in rodents

Following a single challenge with several thousand *N. brasiliensis* larvae, rats develop an immune response that expels the adult worms from the initial infection and prevents re-infection with subsequent challenges. During such a primary

infection with *N. brasiliensis* in rats, three phases are observed corresponding to the initial loss of infective larvae, the establishment and survival of adult worms and the expulsion of the established adult population. The initial loss of larvae is due to failure of between 40 to 60% of larvae to migrate or establish, while expulsion of adults is the result of a developing immune response (Jarrett *et al.*, 1968). Similar infection kinetics are demonstrated by other rodent models of nematode infection such as *Trichinella spiralis* infection of mice and rats (reviewed; Miller, 1984; Rothwell, 1989).

In *N. brasiliensis* infected rats, this developing immune response and expulsion of the challenge may be dose dependent, in a similar manner to that observed in ruminant nematode infections, as a trickle infection regime has been shown to be both cumulative and persistent (Jenkins and Phillipson, 1971). Threshold levels of infection may be tolerated before a response is initiated, as a low number of worms may remain in the gut of "immune" animals following rejection of the bulk of infection. However this population might represent "adapted" worms that avoid the developing host immune response (Jarrett *et al.*, 1968; Rothwell, 1989). This threshold level of infection required prior to an immune response being generated has also been demonstrated in the mouse parasitic nematode *Trichuris muris* (Bancroft *et al.*, 2001).

In a repeat *N. brasiliensis* challenge, loss of larvae is enhanced to over 80%. This is probably due to a specific immune response and represents an "immune exclusion" of incoming larvae from their niche in the small intestine (Miller, 1984). Around 50% of infecting larvae may be lost prior to the lung stages with only 1% of the remaining L4 larvae becoming established as adults. This indicates that components of the immune response operate against both the pre-lung and the gut-establishing larval stages (Love *et al.*, 1974). Expulsion of adult worms also occurs earlier and at a faster rate (Jarrett *et al.*, 1968).

The prevention of establishment of *Nippostrongylus* larvae in immune rats and the expulsion of established adults show similarities with events observed during the development of resistance to *Trichostrongylus* infection in sheep described earlier (Section 1.6.1 Resistance and expulsion in *Trichostrongylus* infection of sheep). The protective immune response against *N. brasiliensis* infection in rats also

shows the same deficiencies with regard to age and reproductive status as does the infection of sheep with *Trichostrongylus*.

Neonatal rat pups infected following birth showed a reduced ability to expel *N. brasiliensis* infection compared to adult rats such that they may harbour significant worm burdens for up to 40 days after infection (Ogilvie and Jones, 1967). The young rats were also unable to mount a protective response to repeated infections until the age of 6 weeks and even at this age the response was not as strong as that expressed in adult rats (Ogilvie and Jones, 1967). In this respect these rats are similar to young lambs, which are unable to mount a protective immune response to nematode infection until around 36 weeks of age (Gibson and Parfitt, 1972). In both infections the lack of response is thought to lie in the lack of development of the immune system.

The phenomena of periparturient relaxation of immunity, similar to that seen in ruminant infection, is also exhibited by lactating female rats infected with *Nippostrongylus* (Houdijk *et al.*,, 2003). Lactating rats that had previously been infected and were therefore immune, harboured higher worm burdens and had higher FEC than non-reproducing counterparts. This observation was also suggested to have its basis in reduced allocation of protein resources as suggested in the ruminant infection (Houdijk *et al.*, 2003).

1.6.3 The immune basis of nematode expulsion

The exclusion of incoming larvae in repeatedly infected animals and the expulsion of adult worms from a single challenge infection result from developing immune responses in both rodents and sheep (Jarrett *et al.*, 1968; Dobson *et al.*, 1990). These are generally characterised as T-helper (Th) 2 responses due to the involvement of specific T-cell subsets and associated cytokines (Finkelman *et al.*, 1997). The specific immune effector responses expressed during GI nematode infection are described in detail in the next section. Presented here is an overview of expulsion events with reference to the underlying immune responses discussed later (Section 1.7 Immune Responses to Infection).

The response of "immune exclusion" expressed against larval stages before they establish in their niche is seen in rodent and in ruminant nematode infections. "Rapid expulsion," was first described in *T. spiralis* infections of rats (reveiwed; Rothwell, 1989) and the term has been used to describe rejection of larvae in other rodent infections including *N. brasiliensis* transplanted adult worms (Miller *et al.*, 1981). It has also been described in experimental *H. contortus* infection (Miller *et al.*, 1983a), and is suggested to occur in field infection of ruminants (Chiejina and Sewell, 1974).

Rapid expulsion is an extremely rapid process by which incoming larvae are removed from the gut within hours of infection. In both *T. spiralis* and *N. brasiliensis* infections, rapid expulsion involves mucus trapping (Miller *et al.*, 1981). In this respect the events in rodent models are similar to the immune exclusion of incoming larvae described in ruminant *Trichostrongylus* infections, that may be the result of antibody / mucus trapping (Harrison *et al.*, 2003).

The expulsion of established adult worms, from primary infected and immune animals, is considered a specific immune-mediated response both in rodent and in ruminant GI nematode infections (Jarrett *et al.*, 1968; Dobson *et al.*, 1990). Similar humoral and cellular responses may be observed in each case (reviewed; Miller, 1984; Balic *et al.*, 2000).

Serum levels of Immunoglobulin (Ig) G, IgA and IgE antibody isotypes may increase during the response, both in rodents and in ruminant infection (Miller, 1984; Balic *et al.*, 2000). During *N. brasiliensis* infection IgE production is particularly strongly stimulated (Jarrett and Haig, 1976) and serum IgG1 is associated with the transfer of passive protection (Jones *et al.*, 1970). Mucosal antibody has been observed in this and other models and may be more important in the resolution of infection (Miller, 1984).

Cellular responses are also stimulated and are implicated in the resolution of infection. Mast cells increase in numbers and tend to aggregate in the infected intestinal tissue areas, correlating with expulsion (Miller, 1984). In some infections, for example during *Strongyloides ratti* and *T. spiralis* infection of mice, mast cells are required for expulsion (Nawa *et al.*, 1994). In others, such as *Nippostrongylus,*

they are strongly stimulated but do not appear to be the effector of expulsion (Miller *et al.*, 1983b; Nawa *et al.*, 1994).

Eosinophilia increases in the blood and eosinophils aggregate at the sites of infection. In addition eosinophils may attach to and damage parasites *in vitro* (McLaren *et al.*, 1977; Shin *et al.*, 2001). Goblet cells may also be involved in the response with an increase in numbers and activation leading to an increased output of mucus into the gut lumen (Karlsson *et al.*, 2000). In *Nippostrongylus* it is this response that is considered to effect worm expulsion (Nawa *et al.*, 1994).

1.7 Immune responses to infection

Expulsion of nematodes from the gastro-intestinal tract and resistance to further infection is mediated by host immune responses. Similar responses are seen in different nematode infections in terms of the cell types, immunoglobulin subtypes and cytokine responses that are expressed. This suggests that there may be common expulsion mechanisms expressed against different nematode species by various host species. However, differences are also evident between different infections indicating that multiple separate immune mechanisms may act on different parasites, life stages and in different host / parasite systems. Termination of an infection might also therefore be brought about by different effector responses in each situation despite general similarities in the responses observed (reviewed Rothwell, 1989; Balic *et al.*, 2000). The similarities and differences of rat and ruminant immune responses to GI nematode infection are summarised in Table 1.2.

1.7.1 T-cells and the immune response

The T-helper cell (CD4+ T-cell) response that develops in response to a pathogen infection is very important in the type of immune response that develops and the subsequent outcome of the infection. T-helper cells are polarised towards T-helper subset 1 (Th-1) or 2 (Th-2) mediated responses (Mosmann *et al.*, 1986). Th-1 responses are described as cell mediated immune responses that are involved with the killing of intra-cellular pathogens (Kaufmann *et al.*, 1992). Th-2 response is described as antibody mediated immune response and involves antibody and different cell types in the resolution of infections with extra-cellular pathogens and epecially helminth parasites (Urban *et al.*, 1992; Finkelman *et al.*, 1997; Liu *et al.*, 2004). That CD4+ T-cell involvement is required for the resolution of GI nematode infection has been demonstrated in rodent and ruminant models by T-cell transfer or depletion experiments (Grencis *et al.*, 1985; Katona *et al.*, 1988; Gill *et al.*, 1993).

In rodent infections with gastro intestinal nematodes, such as *N. brasiliensis, T. spiralis, T. muris* and *Nematospiroides dubius* (formerly *Heligmosomoides polygyrus*), Th-2 polarisation is especially marked and as such these models have been used extensively to study T-cell polarisation (reviewed; Finkelman *et al.,* 1997; Else and Finkelman , 1998). In these models Th-2 reponses are responsible for expulsion while induction of a Th-1 response usually impairs this protection. The idea that all infections are as completely polarised in their T-cell response is now seen as inaccurate with components of Th-1 and -2 possibly involved in a complex regulatory interaction (Allen and Maizels, 1998). However, it has been broadly accepted that immune reactions involving Th-2 like responses are protective during gastro-intestinal nematode infection.

T-cell polarisation is initiated when antigens of the invading pathogen are taken up by antigen presenting cells such as dendritic cells. The antigen molecule is digested into smaller fragments and these fragments are then presented at the cell surface associated with the MHC class 2 protein. A T-cell clone with a specific T-cell receptor then binds the MHC/antigen complex and is activated. Signalling molecules such as cytokines and cell surface molecules are important in this activation and in the subsequent polarisation of response (Else and Finkelman, 1998; Liu *et al.,* 2004). Following activation the T-cell then enters a pathway leading to the production of either a Th-1 or a Th-2 response. The events and interactions of T-cell activation are illustrated in Figure 1.4. Table 1.1 summarises the characteristics of the cell types and the cytokines involved in the T-cell response.

Nematode infections in both rodent and ruminant infection are considered to provoke Th-2 immune responses (Urban *et al.,* 1992; Balic *et al.,* 2000; Schallig, 2000). In this pathway the activated T-helper cells secrete IL-4 and IL-13 generating Th-2 cellular responses (Finkelman *et al.,* 2004) and interact with B-cells presenting the same antigen. This interaction leads to the development of plasma cells and antibody responses, particularly IgE (Figure 1.4).

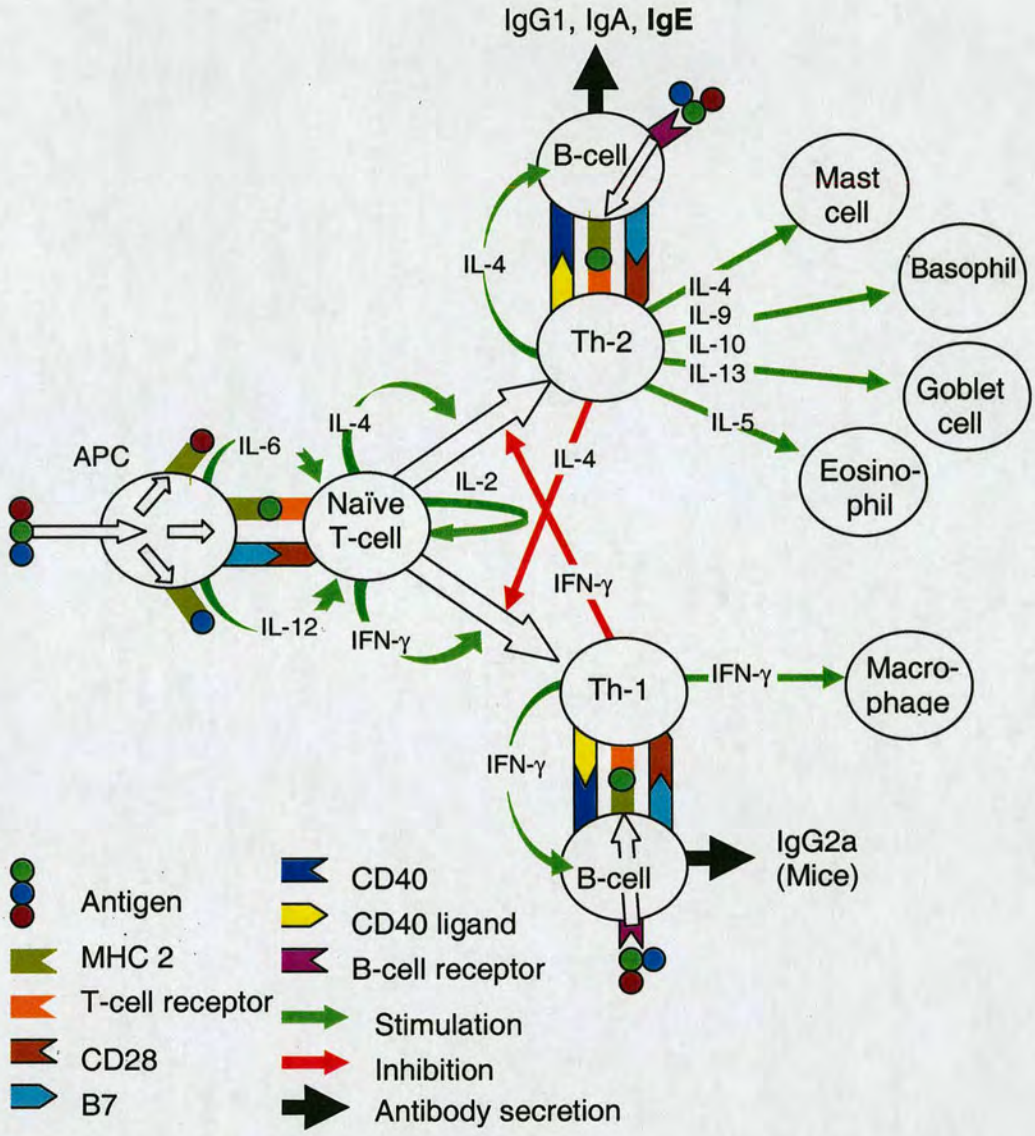


Figure 1.4. The stimulation of T-helper (Th) cell differentiation and the resultant immune responses. Antigen from incoming pathogens is first taken up by antigen presenting cells (APC) and partially degraded. Epitopes are then presented on the cell surface bound to MHC class 2 molecules. Naïve T-cells with T-cell receptor specificity for this MHC 2 / antigen complex are then stimulated by this contact, with CD28 / B7 ligand interaction and cytokine involvement. IL-2 autocrine stimulation leads to T-cell proliferation. The APC contact and cytokine milieu influence the development towards IFN-γ secreting Th-1 like cells or IL-4 secreting Th-2 like cells. Different cytokines are released by each subset leading to the differing cellular immune responses and production of different antibodies through B-cell contact. Negative feedback effects on the development of the opposing subset are displayed by IFN-γ and IL-4. Adapted from Else and Finkelman (1998).

Cell Type	Characteristics	Cytokines secreted (and target)
Antigen Presenting Cells	eg. dendritic cells, macrophages. Take up antigen from pathogens and present epitopes bound to MHC-2. Th-1/2 development may depend on type of APC stimulus.	IL-12 (Th-1 development) IL-6? (Th-2 development)
Naïve T-cell	Resting T-cell. Following activation by APC, proliferation and polarisation of T-cell bearing antigen specific receptor occurs.	Following activation: IL-2 (autocrine stimulation) IFN-γ (Th-1 development; Th-2 inhibition) IL-4 (Th-2 development; Th-1 inhibition)
T helper-1 (Th-1) cell	Release cytokines involved in cell mediated immune responses and induce B-cells to produce Th-1 charateristic antibodies eg. IgG2a in mice. Stimulate macrophages.	IFN-γ (macrophages and B-cells) IL-2 (B-cells)
T helper-2 (Th-2) cell	Releases cytokines involved in antibody mediated immune responses. Stimulate B-cells to produce Th-2 characteristic antibodies eg. IgE.	IL-4 (B-cells, eosinophils, mast cells, basophils, goblet cells and epithelia) IL-10 (B-cells, mast cells and basophils) IL-9 (mast cells and basophils) IL-13 (similar to IL-4) IL-5 (eosinophils)
B-cells	Antigen specific up-take and presentation on MHC-2. On stimulation by activated T-cell proliferate and differentiate into memory and plasma cells. Plasma cells secrete specific antibody.	

Table 1.1. Some characteristics of the cell types involved in the T-cell response and the cytokines released. Through cytokines release (and other signalling molecules) T-cells influence the immune effector response against other infections. During gastro-intestinal nematode infection dominant Th-2 responses are considered protective, whilst dominant Th-1 responses may impede expulsion (Else and Finkelman, 1998; Finkelman *et al.*, 2004).

1.7.2 Cytokine involvement in the immune response

Cytokines are signalling molecules or immune mediators that have many functions in immune reactions including effects on cell recruitment, proliferation and activation. Critically cytokines are involved with the early development of either Th-1 or Th-2 responses and therefore the bias of the immune T-helper cell response. In particular IL-4 and IL-13 are considered cytokines that drive Th-2 development (Finkelman *et al.,* 2004), acting through an IL-4 receptor and the signal tranducer and activation of transcription (STAT) -6 pathway (Hou *et al.,* 1994; Schindler *et al.,* 1994). In contrast Th-1 development is driven by IL-12, acting through the STAT-4 pathway (Bacon *et al.,* 1995; Jacobson *et al.,* 1995) and Interferon (IFN)-γ release (Heinzel *et al.*, 1989). In this regard the production of specific cytokines may be used as markers of Th-1 or -2 response.

The specifc role of individual cytokines in the immune response has been dissected, in part using the previously mentioned rodent / nematode infection models, in which pronounced Th-2 responses occur. Using these models relationships such as the dependance of IgE production and mast cell response on IL-4 and the stimulation of eosinophilia by IL-5 were derived (Coffman *et al.,* 1989; Madden *et al.,* 1991). Some cytokines, their function and target cells in the immune response are summarised in Figure 1.4 and Table 1.1.

The development of many of the responses observed during nematode infection such as the cell types involved and the generation of antibody (and especially IgE) responses are characteristic of Th-2 response. Many of the cytokines observed in rodent nematode infections are also considered Th-2 promoting or characteristic cytokines, as illustrated in figure 1.4 and Table 1.1. These include Interleukins (IL) -4, -5, -6, -10 and –13 (Else and Finkelman, 1998; Behm and Ovington, 2000; Finkelman *et al.*, 2004). Studies in rodent / nematode infection models have shown that the Th-2 promoting cytokine IL-4 is associated with protection whereas the Th-1 promoting cytokine IFN-γ may inhibit rejection (reviewed; Finkelman *et al.*, 1997).

Th-2 cytokine responses have been correlated with immune expulsion of *Nippostrongylus* infection from mouse (Coffman *et al.*, 1989) and rat (Matsuda *et al.*,

1995) hosts. Increases in Th-2 cytokine transcription were measured from the mesenteric lymph nodes during *N. brasiliensis* infection of rats and indicated that IL-3, -4, –5 and –13 mRNA production was elevated, correlating with the time of expulsion (Matsuda *et al.*, 1995; Matsuda *et al.*, 1999). IL-2, -10 and IFN-γ mRNA levels were either unchanged or slightly decreased from controls suggesting an overall Th-2 bias to response. The change in the cytokine levels varied with strain of rat tested (Matsuda *et al.*, 1995). These strains are known to have differing ability to expel worms, such that expulsion is delayed in the strain expressing lower levels of Th-2 cytokines (Uchikawa *et al.*, 1996).

More recently, studies have been conducted into the role of cytokines in ruminant nematode infections (Pernthaner *et al.*, 1997; Gill *et al.*, 2000). These studies indicate changes in cytokine levels being generated during response to ruminant infections with some similarity to those seen in rodents.

Th-2 cytokine production may be upregulated during ruminant nematode infection. IL-4 production increased following immunisation of immune lambs with *T. colubriformis* (Pernthaner *et al.*, 1997). Elevated production of IL-5 mRNA occurred during *H. contortus* infection of lambs, and correlated with a Th-2 immune cell response (Gill *et al.*, 2000). The IL-5 and infiltrating cell responses in this study were greatest from a line of lambs selected for parasite resistance. Further, the level of Th-1 cytokines may be reduced during ruminant nematode infection. The level of Th-1 cytokines IL-2 and IFN-γ was reduced following immunisation of immune lambs with *T. colubriformis* (Pernthaner *et al.*, 1997). IFN-γ production was also reduced during infection with *H. contortus* and was lowest in resistant lambs (Gill *et al.*, 2000). This suggests that the response in ruminants may be Th-2 type with elevated Th-2 cytokines such as IL-4 and -5 and reduced Th-1 such as IFN-γ (Gill *et al.*, 2000).

IL-5 mRNA production by cells from the lamina propria, mesenteric lymph nodes and Peyer's patches in the gut of sheep immune to *T. colubriformis* was associated with eosinophils and γ/δ T cells. The authors ascribe a regulatory role in the activation of effector cells such as eosinophils to this cytokine (Bao *et al.*, 1996). IL-6 mRNA producing cells in the intestine of sheep also increased in number during infection with *T. colubriformis* (Shen *et al.*, 2000). This increase was correlated with

increases in immunoglobulin containing cells and specifically IgG containing cells. The authors suggest a role in the increase of IgG production to this cytokine response (Shen *et al.*, 2000).

1.7.3 Mast cells, basophils and globule leukocytes

Mast cells are an immune effector cell commonly associated with nematode infections. They release mediators from cytoplasmic granules including histamine, proteoglycans, cytokines and proteases that have effects on the local tissues. Mast cells fall into two main types, connective tissue and mucosal, and derive from the same stem cell line as basophils, containing similar products and activation factors including high affinity IgE receptors. Basophils circulate in the blood stream whereas mast cells are localised to tissue sites and their differentiation is under the control of different cytokines (Balic *et al.*, 2000). Globule leukocytes (GLs), which are often referred to in literature, are now believed to be intra-epithelial mast cells with enlarged granules. These may be the effector stage ready to release the granule components (Rothwell, 1989; Balic *et al.*, 2000).

During nematode infections, mast cells proliferate and infiltrate the site of infection (mastocytosis). They then degranulate releasing the contents into the surrounding tissue environment or intestinal lumen. Consequent effects may include increased vascular permeability, mucus secretion and smooth muscle contraction as well as recruitment of other immune cells and effector mechanisms (Balic *et al.*, 2000).

The level of one mast cell product, mast cell protease (MCP), rises systemically and in mucosal tissue during both rodent and sheep nematode infections, in both cases an event correlating with expulsion (Miller *et al.*, 1983b; Huntley *et al.*, 1993; Douch *et al.*, 1996). MCP level is therefore a useful marker of mast cell activation during nematode infection. It is thought that MCP may act on epithelial cell tight junctions to increase the epithelial permeability (Miller, 1984; McDermott *et al.*, 2003).

Numbers of mast cells and GL increase in the small intestine mucosa during infection and expulsion of *Trichostrongylus* infections in sheep (O'Sullivan and Donald, 1973), and guinea pigs (Huxtable and Rothwell, 1975). Lumenal and tissue GL numbers also increase with resistance status following repeated and abrogated larval challenge with *T. colubriformis* (Stankiewicz *et al.*, 1993). Mast cell and, in particular, GL numbers correlate negatively with numbers of adult worms in the intestine during *Trichostrongylus* infection of sheep, suggesting a correlation with protection (Douch *et al.*, 1986). The increase in numbers of GL during infection also correlated positively with increased larval migratory inhibitory action of mucus in infected sheep (Stankiewicz *et al.*, 1995)

Treatment with dexamethasone, a corticosteroid, abrogated the rejection of GI nematodes in the sheep. There was a corresponding reduction in serum MCP level and tissue GL number. Both effects ended when the drug treatment was removed (Douch *et al.*, 1996).

The rise in effector cell number varies with the reproductive status of infected ewes, a factor that influences ability to resist infection. This may suggest that reduction in effector cell accumulation, including mast cells, is a factor in the loss of immunity of periparturient ewes (O'Sullivan and Donald, 1973).

Mastocytosis is very pronounced during *Nippostrongylus* infection of the rat (Kelly and Ogilvie, 1972; Miller *et al.*, 1983b). Mast cells and GL accumulate in infected areas in correlation with the time of expulsion, (Kelly and Ogilvie, 1972). Degranulation of activated mast cells, as determined by increase in systemic level of MCP, occurs at around the time of worm expulsion (Miller *et al.*, 1983b). These observations might suggest that the mast cell response is a component of the expulsion in the *N. brasiliensis* infection, by a mechanism such as the increase in mucosal permeability (Miller, 1984).

In several other parasite models, including *S. ratti* (Nawa *et al.*, 1985) and *T. spiralis* (Ha *et al.*, 1983; McDermott *et al.*, 2003) in mice, mast cell activation has been linked to the expulsion of the worms. In *Nippostrongylus* infection of mice however the expulsion of worms occurs even in the absence of a mast cell response, or a mast cell component (Uber *et al.*, 1980). It has also been suggested that *N. brasiliensis* expulsion is a consequence of goblet cell activation following

observations by some workers (Ishsikawa *et al.*, 1994; Nawa *et al.*, 1994). This would suggest that while strong stimulation of mast cell accumulation and activation occurs during *N. brasiliensis* infection, this is the result of a generalised response against nematode infection and not the specific effector (Nawa *et al.*, 1994). MCP is however a good indictor of mast cell activation and therefore of a cellular component in the response during *N. brasiliensis* infection (Miller *et al.*, 1983b).

1.7.4 Eosinophils

Eosinophils are a cytotoxic immune cell type commonly associated with parasitic infections. They are produced in bone marrow and circulate in the blood with numbers increasing and accumulation occurring at sites of infection. Eosinophils have granules containing strongly cationic proteins including peroxidase (eosinophil peroxidase) and lyosomal proteins, which are released onto pathogens by activated eosinophils, and which are capable of a respiratory burst that produces reactive oxygen species (Babior *et al.*, 1973). During nematode infection, eosinophils accumulate in infected tissue and eosinophil association with dead or damaged worms in some systems has suggested they may be involved in worm killing (Rothwell, 1989; Balic *et al.*, 2000).

Studies with nematode larvae *in vitro* in several species including *Nippostrongylus* (Shin *et al.*, 2001) and the ruminant nematode *H. contortus* (Rainbird *et al.*, 1998) have demonstrated that eosinophils are capable of attaching to, degranulating and damaging or killing nematode larvae. These studies also showed that larval killing by eosinophils *in vitro* is enhanced by the addition of antibody, complement and IL-5, which is indicative of an *in vivo* relationship (Rainbird *et al.*, 1998; Shin *et al.*, 2001).

In the guinea pig *T. colubriformis* infection system, eosinophilia and increased mast cell counts coincide with worm expulsion (Huxtable and Rothwell, 1975; Rothwell, 1975). Increased tissue eosinophil numbers have also been correlated with resistance in ruminant nematode infection suggesting a role in protection (Bao *et al.*, 1996). In a mixed *T. colubriformis / H. contortus* infection

eosinophil numbers, along with mast cells and GL were increased in the gut mucosa of animals with reduced faecal egg outputs (O'Sullivan and Donald, 1973).

Circulatory eosinophil counts were also correlated with increased protection in a line of sheep that had been bred for resistance to *T. colubriformis* (Dawkins *et al.*, 1989) and in goats with increased resistance to *T. circumcincta* and *T. vitrinus* (Patterson *et al.*, 1996). The eosinophilia was more pronounced and the FEC were lower in resistant groups than in controls with low resistance to infection in both studies. Another study, however, showed that circulatory eosinophilia was not always more pronounced in *H. contortus* or *T. colubriformis* resistant animals, possibly due to differences in infection regimes, and therefore might not be useful as an indicator of increased resistance (Woolaston *et al.*, 1996).

1.7.5 Goblet cells and mucus

Goblet cells are specialised mucus producing cells present in the epithelium of the intestine and also the respiratory tract. The cells are largely filled with mucinogen the precursor of mucus, which gives the cells a characteristic goblet shape. Mucus secretion from crypt goblet cells may be rapidly and dramatically increased under the influence of parasympathetic nerves in the intestine (Specian, 1980). Goblet cell hyperplasia occurs during nematode infection (reviewed; Rothwell, 1989).

During *N. brasiliensis* infections goblet cell hyperplasia occurs and both the amount of mucus increases and its glycosylation changes (Karlsson *et al.*, 2000). This mechanism has been strongly linked to the expulsion of *N. brasiliensis* adult worms and has been suggested as the effector mechanism of expulsion in this infection (Nawa *et al.*, 1994).

Ishikawa *et al.*, (1994) suggested that adult *N. brasiliensis* induce mucus secretion and are expelled only after they have become damaged by the host immune response. "Normal" or undamaged worms do not induce mucus secretion and actively inhibit it by means of excreted or secreted (ES) products. Mucus secretion may be increased by acetylcholine from parasympathetic nerves in the intestine

(Specian, 1980). *N. brasiliensis* secretes copious amounts of acetylcholinesterase (AChE) which might act as a defence against immune response mechanisms (Sanderson and Ogilvie, 1971). This secreted AChE is recognised by the host antibody response against *N. brasiliensis* and the AChE isoform profile changes during infection as the worms become "damaged" (Jones and Ogilvie, 1972). This suggested mechanism of expulsion might then fit the observed data.

Goblet cell numbers also increase during *T. colubriformis* infection in guinea pigs and sheep (Manjili *et al.*, 1998). In guinea pig strains bred for resistance, earlier hyperplasia and changes in mucin sulphation, similar to those seen during *Nippostrongylus* infection in the rat, were associated with resistant strains (Karlsson *et al.*, 2000). Goblet cell accumulation did not however correlate with protection during infection in sheep (Douch *et al.*, 1986).

While goblet cell accumulation has not been linked to resistance in *Trichostrongylus* infections of sheep, mucus itself is thought to have a protective role. Trapping of nematode larvae in mucus from sheep immune to *T. colubriformis* has been demonstrated *in vitro* (Douch *et al.*, 1986; Stankiewicz *et al.*, 1995) and *in vivo* (Harrison *et al.*, 1999). This activity was correlated to the number of GL and the aryl sulphatase content of the mucus in the lumen (Stankiewicz *et al.*, 1995). Specific antibody in mucus may also aid in binding antigen and complexing with mediators in the mucus (McClure *et al.*, 1992; Harrison *et al.*, 2003).

1.7.6 Antibody responses

Antibody responses are generated against parasite antigens by the host following nematode infection. Parasite specific IgG, IgE and IgA isotypes have been demonstrated in responses against GI nematodes in various species, both to primary and secondary infections (reviewed; Miller, 1984; Balic et al, 2000). Specific IgG levels can increase in the serum and also in GI mucus. IgG1 isotype tends to predominate over IgG2 in nematode infections, indicative of the Th-2 immune bias (Balic *et al.*, 2000). IgE production is stimulated by infection and may be induced by worm secreted allergens (Jarrett and Miller, 1982). IgE increases are associated with allergic and hypersensitivity type responses and may be involved in mast cell activation by cross-linking with antigen (Shaw *et al.*, 1998). IgA is associated with antibody responses at mucosal surfaces. Specific IgA responses may be generated at the site of infection in the GI mucosa during nematode infections (Miller, 1984). Responses to secondary infections tend to occur earlier and peak sooner than those elicited by primary infections due to anamnestic responses primed by the primary infection and triggered on secondary challenge (Shaw *et al.*, 1998).

T. colubriformis-specific antibody is produced both locally and systemically on challenge of immune animals (Adams *et al.*, 1980). Sheep vaccinated with repeated *T. colubriformis* larval infections subsequently rejected a challenge infection and showed raised serum titres of IgG1, IgG2 and IgA compared to unvaccinated controls (McClure *et al.*, 1992). Early rejection of incoming larvae was associated with increases in worm-specific IgG1 and IgG2 in intestinal mucus. Rejection of the remaining worms was associated with increased IgG2 and IgA isotypes in the mucus (McClure *et al.*, 1992).

The IgG1 and IgM responses to both adult and L3 antigens were greater in a line of sheep selected for reduced FEC than in an unselected line (Bisset *et al.*, 1996). Animals with higher antibody titres had higher levels of protection, suggesting a correlation of immunoglobulin level with protection, and implicating antibody as an effector of this protection.

The IgE response during *T. colubriformis* challenge has been examined using enzyme linked immunosorbent assay (ELISA; Shaw *et al.*, 1998). Serum levels

conformed to the general pattern, rising slowly during primary infection and more sharply during secondary. The IgE response in this study was also correlated with the mast cell response and the onset of immunity to infection during both primary and secondary challenges. IgE has been linked to mast cell activation and mast cells express high affinity IgE receptors on their cell surface (Ishizaka *et al.*, 1975). The correlation with IgE and mast cells may indicate a role for IgE in direct or mast cell-mediated termination of *T. colubriformis* infection (Shaw *et al.*, 1998).

Antibody responses have been demonstrated during infection with *N. brasiliensis* (reviewed; Ogilvie and Jones, 1971). In particular, *N. brasiliensis* is known to induce large specific and non-specific IgE responses (Jarrett and Haig, 1976; Yamada *et al.*, 1993a). The IgE response is associated with and may be induced by secreted or excreted allergens (Yamada *et al.*, 1991; Uchikawa *et al.*, 1993). Specific IgE responses are linked to amine release from mast cells *in vitro* and *in vivo* although this has been shown on some occasions not to correlate with protective immune responses (Ogilvie and Jones, 1971).

Increased levels of IgG1 and IgG2, IgA and IgM have also been reported during infection, although the increases were modest compared to those seen in IgE (Jarrett and Bazin, 1977). IgG1 responses occur to some similar antigens to those responsible for stimulating IgE upregulation in *Nippostrongylus* ES (Yamada *et al.*, 1993b; Kamata *et al.*, 1995; Nakazawa *et al.*, 1995). IgG2a responses, however, were stimulated by a different group of antigens present on the adult worm (Yamada *et al.*, 1993b). Experiments have implicated IgG1 as the antibody responsible for the passive transfer of protection in this system (Jones *et al.*, 1970).

In their review of *Nippostrongylus* expulsion, Ogilvie and Jones (1971) considered worm expulsion to be a two-stage phenomenum. The worms first being damaged by antibody, mainly of the IgG isotype with a further mechanism, at that time unexplained, leading to the expulsion of damaged worms. This mechanism of expulsion has been backed up by a later investigation, which suggested that changes in mucus secretion removed the worms, following damage by an immune-mediated response (Ishsikawa *et al.*, 1994).

Antibody / mucin interaction has been implicated in the trapping of worms during *T. spiralis* infection of rats (Carlisle *et al.*, 1990). This is an interesting

observation given the implicated role of mucus in the mechanism which has been suggested to remove *Nippostrongylus* from immune rats and the larval inhibitory action of mucus in sheep *Trichostrongylus* infections (Miller, 1987; Ishsikawa *et al.*, 1994).

Rodent / *Nippostrongylus*	Ruminant / nematode
T-cell requirement for expulsion in rats (Keller and Keist, 1972) and mice (Katona *et al.*, 1988).	T-cell requirement for expulsion of infection (Gill *et al.*, 1993)
Th-2 cytokines are up-regulated and Th-1 cytokines are down-regulated during expulsion in rats (Matsuda et al, 1995). Th-2 cytokines (IL-4, IL-13) lead to expulsion while Th-1 cytokines (IL-12, IFN-γ) lead to persistence in mice (Else and Finkelman, 1998; Finkelman *et al.*, 2004).	*Th-2 cytokine increases and Th-1 reductions may correlate with protection but results are equivocal (Pernthaner et al., 1997; McClure et al., 1995; Balic et al., 2000)*
Response seen against early tissue migratory phases in lung (Matsuda *et al.*, 2001).	*No tissue migratory phase*, however response observed to tissue associating L3/L4 in gut (Emery *et al.*, 1992)
Mast cell accumulation and degranulation correlated to protection (Kelly and Ogilvie, 1972; Miller *et al.*,1983b).	Mast cell accumulation and degranulation correlated to protection (Douch *et al.*, 1986; Douch *et al.*, 1996).
Eosinophil response correlated to protection and kill infective larvae (Kelly and Ogilvie, 1972; Shin *et al.*, 2001)	Eosinophil response correlated to resistance and kill infective larvae (Bao *et al.*, 1996; Rainbird *et al.*, 1998).
Goblet cell activation / mucus trapping responsible for expulsion (Nawa *et al.*, 1994).	*Goblet cell hyperplasia not correlated to protection,* but mucus trapping of larvae reported (Douch *et al.*, 1986).
IgE, IgA, IgG responses correlated to protection (Jarrett and Haig, 1976; Jarrett and Bazin, 1977).	IgE, IgA, IgG responses correlated to protection (McClure *et al.*, 1992; Shaw *et al.*, 1998).
IgG1 responsible for passive transfer of protection (Jones *et al.*, 1970).	*IgG1 predominates over IgG2 during nematode infections (Balic et al., 2000).*
IgG subtypes IgG1 and IgG2a linked to Th-2 response. IgG2b and c linked with Th-1 response (Cetre *et al.*, 1999).	*IgG1 associated, but not definitively shown to be Th-2 linked, while IgG2 considered Th-1 (Gill et al., 2000).*

Table 1.2. Some similarities *and differences* between the immune response of the proposed model and that of nematode infected ruminants. Similarities include the nature of the cell types and antibody involved. There is less evidence in ruminant infections of the marked Th-1 / Th-2 dichotomy observed in rodent models and the proposed mechanism of expulsion may differ. The tissue migratory phase of *Nippostrongylus* infection also represents a difference in the life cycle leading to differences in the observed immune responses

1.8 Methods for the control of nematode parasites

Due to the substantial production and economic losses that can be caused even by sub-clinical infections, the control of nematode GI infections in livestock is both important and necessary (Kyriazakis *et al.*, 1996). The current methods used for control of ruminant GI nematodes are based mainly on the use of chemical agents with some use of pasture management. Frequent treatment and prophylactic dosing of animals with anthelmintic drugs has been the most effective and primary means of control.

1.8.1 Anthelmintic Drugs

Livestock, both sheep and cattle, are routinely dosed, or drenched, with anthelmintic drugs in regimes designed to suppress nematode infection and limit production loss and clinical infections. Dosing at around 4 weeks before parturition and 4-6 weeks after lambing is recommended for ewes and 2-3 times following weaning for lambs depending on how intensive the stocking rate is (Urquhart *et al.*, 1987). Three classes of anthelmintic are currently in common use, namely the benzimidazoles, levamisoles and macrocyclic lactones (avermectins). These are routinely administered orally using a drenching gun in a liquid formulation although some development of slow release bolus formulations has been carried out (Donald, 1985).

Mechanisms of drug action

The molecular and biochemical modes of anthelmintic action of several of the different classes have not, as yet, been absolutely defined. Different cell processes are thought to be affected by the different drugs.

Benzimidazoles have been shown to interfere with parasite microtubule formation (Lacey and Prichard, 1986; Lacey and Gill, 1994; Kwa *et al.*, 1994). The

drug binds to the β-tubulin subunit and prevents aggregation thereby preventing formation of the microtubules. Interference with this major cell structure protein causes the death of the parasite (Prichard, 1990).

Avermectins are considered to act through a high affinity receptor that leads to an increase in membrane Cl^- permeability. They have been shown to act as ligands on a glutamate gated chloride channel (Yates *et al.*, 2003). This activation causes hyper-polarisation of the membrane and thus interferes with nerve and muscle cell polarisation. In *Caenhorrabditis elegans, H. contortus* and *T. colubriformis* the drug causes interference with pharyngeal pumping and feeding while fecundity effects and paralysis of the worms also result (Sheriff *et al.*, 2002; Yates *et al.*, 2003).

Levamisoles act as cholinergic agonists on nicotinic acetylcholine receptors, which activate cation channels in somatic muscle (Martin *et al.*, 1998). Depolarisation and contraction of the muscles leads to a spastic paralysis of the nematodes facilitating their removal (Atchison *et al.*, 1992).

Problems with control of nematode parasites using anthelmintic drugs

Although they are the primary choice and currently the most effective means of control, there are problems with the use of anthelmintic chemicals. Resistance of nematodes to the drugs threatens the long-term sustainability of this means of control. Concerns over drug residues in food products and the growth of the organic sector has increased consumer pressure to reduce their use.

Due to widespread use and long term application, resistance of the parasites to all classes of drugs currently available has been identified. The use of benzimidazole anthelmintics selects for worms already present in the population and carrying a mutation in the β-tubulin gene conferring drug resistance (Kwa *et al.*, 1994). Molecular mechanisms of resistance to the other classes of drug have not as yet been defined with certainty, but may involve changes in the gama-aminobutyric acid (GABA) receptor (Blackhall *et al.*, 2003) and P-glycoproteins (Kerboeuf *et al.*, 2003).

In some areas drug resistance is widespread and parasites resistant to several drug classes are present. This problem is so severe that it has major effects on the sustainability of livestock production in some areas of the world (Waller *et al.*, 1996; van Wyk *et al.*, 1997).

In the UK parasites resistant to benzimidazoles, levamisoles and ivermectin have been identified, with benzimidazole resistance being particularly widespread. Resistance to these drugs is thought to be spreading in parasite populations (Mitchell *et al.*, 1991; Bartley *et al.*, 2003).

1.8.2 Pasture management

Pasture management techniques may be employed as a control measure to prevent or minimise infection (Barger, 1997). Eggs and larvae of most nematode species have a life span on pasture limited to around one year (apart from *Nematodirus sp.*) and moving animals onto fresh pasture with a suitable gap or fallow period will reduce the larval challenge.

Systems involving moving animals onto fresh pastures at strategic times can maintain exposure at sub-clinical levels during critical periods such as the weaning of lambs. Management of grazing in this way may be used in conjunction with anthelmintic treatment to limit the infection of the "clean" pasture with nematode eggs (Lindqvist *et al.*, 2001). Alternatively, rotating sheep with cattle on pasture may be an effective method of controlling parasite infection by preventing the build up of host-specific species on mixed herd farms (Barger, 1997). Moving animals to clean pasture in this way may be used as part of a strategy to reduce anthelmintic use. Alternatively, a pasture moving strategy may be effective enough at controlling parasite infection to be used without drugs as a means of limiting clinical infection (Githigia *et al.*, 2001).

There are several problems with this approach. Climatic variables play a great role in the suitability of such a strategy. Temperature and rainfall influence the longevity of eggs and larvae on pasture as well as determining which nematode species will be present. While wet tropical climates might be more suited (Barger *et*

al., 1994), temperate climates may be unsuitable for such a strategy due to the long survival time of the larvae (Gibson, 1973).

The frequency of moving animals may be limited by the amount of pasture available. Animals must be moved at an interval such that nematode numbers do not increase while at the same time pasture has had sufficient time to be decontaminated. Limited pasture and high stocking densities may be limiting factors in such a system and as such these strategies do not always lend themselves to intensive farming practices (Uriarte and Valderrabano, 1990).

Pasture management is often not effective enough to limit parasite infection on its own. Some grazing systems only limit the infection to a degree necessary to reduce anthelmintic use and not completely stop its use (Barger, 1997). Pasture management strategies may also increase the selection pressure towards parasite genotypes or species that are least affected by this strategy (Barger, 1997). This would therefore ultimately reduce the effectiveness of such a strategy in the long term. Such issues would need to be considered in the design of control programs based on pasture management (Barger, 1997).

1.9 Novel control strategies

The problems with current control strategies have led to an interest in the development of novel, non-chemical methods of parasite control. Several approaches including biological control with fungi, genetic selection of nematode resistant sheep and vaccination are being researched.

1.9.1 Larval trapping fungi

Several studies have investigated the use of nematophagous fungi for the control of nematode parasites on pasture (Larsen, 1999). Species of fungi, including genera *Duddingtonia* and *Arthrobotrys,* actively trap and feed on nematode larvae in faecal pats deposited on pasture. Some of these species can survive passage through the gut of ruminants (Larsen *et al.*, 1992). By using fungi as a food additive it has therefore been possible to reduce larval counts in faecal cultures collected during sheep trials (Faedo *et al.*, 1997). Pasture larval contamination has also been demonstrably reduced in both sheep (Faedo *et al.*, 1998) and cattle trials (Wolstrup *et al.*, 1994), indicating the potential of this approach in nematode control.

In these trials animals were dosed with set numbers of fungal chlamydospores. During trials in which individual animals were allowed free access to fungi as a food additive, differences in uptake rates occurred (Jackson *et al.*, 2003). Differences in larval survival in differing climates might also reduce the effectiveness of the treatment (Gibson, 1973). Therefore optimisation of conditions and delivery systems must be carried out before biological control with fungi becomes viable.

1.9.2 Selective breeding

Differences in susceptibility to nematode infection or clinical disease caused by infection exist both between and within sheep breeds (Burke and Miller, 2002;

Gauly and Erhardt, 2001). Some of this difference is accounted for by genetic difference between animals. Farming more resistant breeds in areas of high incidence of nematodiasis is one method of exploiting this genetic resistance to infection. However breeds that are more resistant to nematode infection might be less suitable in other essential traits such as wool production, carcass quality and weight gain.

Selectively breeding from animals more resistant to infection within established breeds can increase the disease resistance status of the offspring (Gauly and Erhardt, 2001). Selective breeding for nematode resistance in this way has been predicted to have the potential to reduce production loss (Windon, 1990).

Selective breeding schemes within breeds of sheep (Bisset *et al.*, 1996; Kahn *et al.*, 2003) and goats (Patterson *et al.*, 1996) have been carried out with some success in generating lines resistant and susceptible to parasite infection. By using FEC and other markers of response, it has been possible to select rams and dams with a resistant phenotype as breeding stock and thus generate more resistant offspring down several generations (Bisset *et al.*, 1996; Kahn *et al.*, 2003). This approach has been successful to the point of being commercially applicable in Australia and New Zealand (Gray, 1997).

Breeding for increased resistance however may not be the most suitable strategy, if resistance is not accompanied by a production gain. In a selective program, production traits would need to be maintained, or improved, along with improved resistance. This may not always be the case, due to complex genetic heritability and linkage of traits. Unsuitable traits, such as breech soiling, may also be increased in selected lines (Bisset *et al.*, 1997). The assessment of production parameters is therefore an important part of a selective breeding program. Breeding for tolerance of infection, whereby lambs have improved weight gain under infectious conditions, rather than focussing on reducing FEC may be a more suitable strategy (Bisset and Morris, 1996). Given the central role of Th-2 responses in worm expulsion, selection for nematode resistance may have the effect of increasing susceptibility to viral and protozoal infections where a Th-1 response is desirable for disease control.

1.9.3 Improved nutrition and dietary supplementation

Improved nutrition of animals can enhance the immune response to infection and has a direct effect on the clinical expression of disease and productivity during infection (Kahn *et al.*, 2003). Increasing the quality of the diet can severely reduce the weight loss effects and reduce FEC and worm burden during infection with *T. colubriformis, H. contortus* and other nematodes. In particular, the protein content of the diet as opposed to the total energy appears to be of importance (Kahn *et al.*, 2000; Strain and Stear, 2001).

Limited resources for feeding sheep and increased production costs may limit this approach for commercial or extensive farming. Nutritional supplementation could have utility for organic / pedigree animal production where increased cost is more acceptable for an improvement in animal quality or in intensive systems where animal are fed on pelleted diets.

Addition of substances to the diet that adversely affect the worms has also been investigated. Dietary supplementation with plant foods rich in tannins can decrease adult worm burden and FEC (Athanasiadou *et al.*, 2001). Tannins may have an effect on the worms directly or enhance the uptake of nutrients in the ruminant digestive tract (Niezen *et al.*, 1998).

Copper wire particles, when given as a feed supplement, have been shown to reduce *H. contortus* infection (Knox, 2002). Molybdenum has also been administered experimentally as a dietary supplement that reduces worm burden, probably through a mechanism involving free radical generation (Suttle *et al.*, 1992a; 1992b). Either of these supplements may be toxic at increased levels and so may not be useful practically or require careful dose limitation.

1.10 Vaccination

Host immunity to re-infection develops in ruminant GI nematode infections including *Trichostrongylus* and also to *N. brasiliensis* in rats. This suggests that, the host expresses protective immune responses and that protection could be elicited by vaccination with parasite material. Several different strategies of vaccination have been attempted or are being investigated.

1.10.1 Attenuated larval vaccines

Early attempts at nematode control by vaccination were by the use of live attenuated larvae. Larvae can be attenuated by gamma irradiation and will then produce self-limiting infections that give significant levels of protection to re-infection. The main success of this approach was the development of a live attenuated vaccine against *Dictyocaulus viviparus*, the bovine lungworm, which remains the only effective and commercially available nematode vaccine (Jarret *et al.*, 1958).

Protection levels of 97-99% were also generated against *T. colubriformis* infection in sheep, by vaccination with 20,000 irradiated L3 larvae (Gregg and Dineen, 1978). The number of larvae required was however prohibitive, as several donors would be required to provide a single protective dose. The treatment also failed to protect young lambs, the most important target group for a control strategy (Emery *et al.*, 1993).

1.10.2 Native and recombinant antigens

The advent of recombinant protein technology has provided a potential new source for parasite antigens (Knox, 2000; Knox *et al.*, 2001). Research has concentrated on the isolation of parasite proteins, which protect the host against infection, with the aim of producing them *in vitro* as recombinant antigens. A

"fractionate and vaccinate" approach has been taken in order to isolate protective fractions, and protective proteins from several sources of parasite material such as parasite tissues or parasite ES proteins (Smith, 1999). Separating and identifying individual proteins from such complex mixtures is difficult and time consuming, although some success has been achieved. Examples of helminth vaccines using this approach are the cestode recombinant protein vaccines derived from onchosphere surface tissues and the "hidden" nematode antigens discussed below (Smith, 1999).

Genomic tools such as cDNA libraries and expressed sequence tags (ESTs) are also used as a source from which to select vaccine targets. Selecting immuno-reactive, highly or differentially expressed proteins from such datasets is one method that may be used to select potential vaccine targets for recombinant expression (Knox *et al.*, 2001).

1.10.3 Hidden antigens

One approach that has been particularly successful is the targeting of gut expressed, or hidden, antigens in blood-feeding nematodes such as *H. contortus*. These antigens are mostly integral membrane proteins, often enzymes thought to be associated with the digestion of the blood meal. The vaccination strategy depends on the parasite ingesting significant quantities of immunoglobulin as they feed, the ingested specific antibodies bind to the proteins in the worm's gut and inactivate them, thus preventing normal function and killing the worms (Newton and Munn, 1999; Knox and Smith, 2001). A recombinant vaccine against the cattle tick *Boophilus microplus* based on this principle has already been developed commercially in Australia (Willadsen *et al.*, 1995).

Vaccination studies against *H. contortus* are well advanced with several candidate, hidden antigens having been identified including H11 and H-gal-GP. These enzyme complexes were identified using a "fractionate and vaccinate" approach from gut tissue dissected from adult worms (reviewed Newton and Munn, 1999; Knox and Smith, 2001).

Other species of nematode including *Trichostrongylus sp.* and *T.circumcincta* are, however only partially susceptible to this approach, as they do not feed primarily on blood. They do not, therefore, ingest sufficient quantities of immunoglobulin to inhibit digestion (Knox and Smith, 2001).

1.10.4 Excreted / secreted antigens

Many proteins are excreted or secreted into their environment by nematode parasites at different life stages (Pritchard, 1986). Some of these proteins are functional and have been ascribed roles in parasite feeding, tissue penetration or immune evasion. Some ES proteins are also immunogenic. Therefore ES proteins are rational vaccination targets and studies have examined ES as a source of protective antigens.

Protective nematode ES antigens have been identified, including 15 and 24 kDa proteins from the nematode *H. contortus* (Schallig *et al.*, 1997a; 1997b). These proteins shared homology with two ES proteins of *T. colubriformis,* already identified, which induced protective responses in Guinea pigs (Savin *et al.*, 1990; Dopheide *et al.*, 1991). The larger protein also shared homology with an *Ancylostoma caninum* secreted protein of interest (Schallig *et al.*, 1997b).

Immunisation with Cathepsin L proteases from the ES of the ruminant trematode parasite *Fasciola hepatica* also gave protection in a trial in cattle (Dalton *et al.*, 1996). This was associated with the generation of specific antibody responses (Mulcahy and Dalton, 2001).

ES proteins have been implicated in the persistence of some intestinal nematodes in their host and have functional homologues in other species occupying the same niche. These proteins may protect the parasite from host immune responses and hence are suitable targets for immunisation. Two such proteins of particular interest in the present study are superoxide dismutase (SOD) and AChE, which are both released *in vivo* by nematode GI parasites of interest.

1.10.5 Superoxide dismutase

Superoxide radicals generated by host leukocytes are part of the immune response and can damage nucleic acids, proteins and membranes. Superoxide radicals such as O_2^- and OH^- as well as oxidative responses, have been implicated in resolution of infections with *N. brasiliensis, T. vitrinus* and *H. contortus* and other nematodes (Henkle-Duhrsen and Kampkotter, 2001).

Oxidative responses occur during a *N. brasiliensis* infection and adult worms are damaged by oxygen radicals *in vitro* (Smith and Bryant, 1986). Administration of antioxidants that are active in the gut of rats will delay expulsion of *N. brasiliensis in vivo* (Smith and Bryant, 1989a). Similarly in sheep and lambs dietary supplementation with molybdenum, a chemical involved in free radical generation, reduced the total worm burdens during *H. contortus* and *T. vitrinus* infections (Suttle *et al.*, 1992a; 1992b).

Eosinophils are implicated in the host response to *Nippostrongylus* (Shin *et al.*, 2001) and *Trichostrongylus* (Dawkins *et al.*, 1989). Eosinophils contain peroxidase in their granule proteins and are capable of a prolonged respiratory burst generating oxidative radicals (Babior *et al.*, 1973). Eosinophils have also been demonstrated to attach to and release their granule proteins on the surface of nematode larvae *in vitro* (Rainbird *et al.*, 1998; Shin *et al.*, 2001). It is possible that the generation of oxidative responses by eosinophils *in vivo* is one mechanism by which they could damage nematode parasites.

SOD and several other enzymes including catalase, glutathione peroxidase and a novel family of peroxiredoxins are involved in pathways that protect the parasite from free radical damage, as shown in Figure 1.4 (Henkle-Duhrsen and Kampkotter, 2001). SOD converts the superoxide radical, O_2^-, to H_2O_2, one step in this enzymatic process. Some evidence suggests that SOD is released by nematode parasites including *N. brasiliensis, T. vitrinus, H. contortus* and *T. circumcincta* (Knox and Jones, 1992; Hadas and Stankiewicz, 1998). This has received further support by the demonstration that nematodes, for example *H. contortus,* express mRNA transcripts encoding both cytosolic and extracellular forms of the enzyme

(Liddell and Knox, 1998). Increased levels of SOD have been correlated with persistence of *N. brasiliensis* in the rodent host (Smith and Bryant, 1989b).

As noted above, *H. contortus* expresses two distinct SODs, a cytosolic and a putative extra-cellular form. A vaccination trial with *H. contortus* SOD showed a 17% reduction in parasite burden, a low but significant level of protection. This was achieved using a systemic vaccination with single challenge infection (Liddell and Knox, 1998). Further investigation of immunisation with SOD is an aim of this project, specifically investigating the efficacy of SOD vaccination against a low-level parasite challenge and comparing the outcome of systemic and mucosal vaccination.

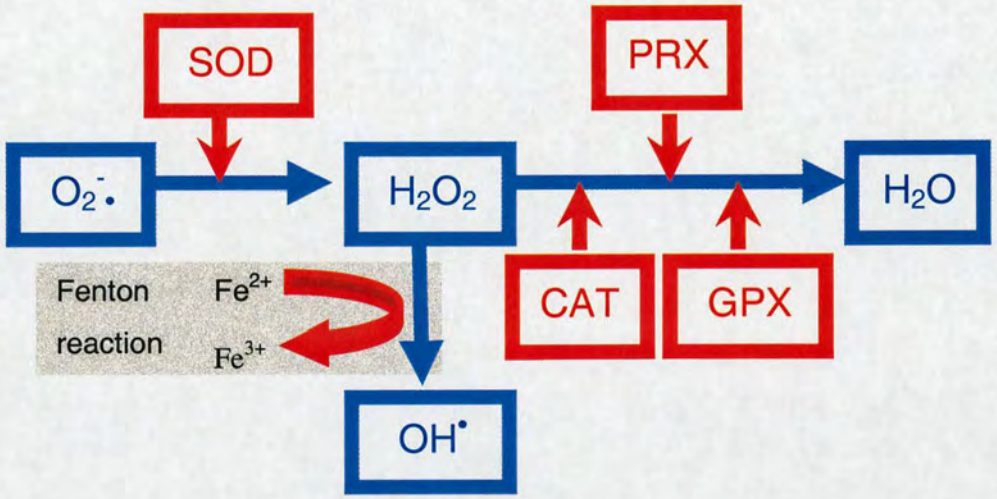


Figure 1.5. Antioxidant pathways (blue) and antioxidant enzymes (red) of parasitic nematodes (adapted from Henkle-Duhrsen and Kampkotter, 2001). Oxygen radicals are converted to harmless products under the influence of these enzymes. Such reactions may be important in the protection of the nematodes from host mediated oxidative immune responses. SOD = Superoxide dismutase; PRX = Peroxiredoxin; CAT = Catalase; GPX= Glutathione peroxidase.

1.10.6 Acetylcholinesterase

The enzyme AChE is secreted in large amounts by *N. brasiliensis*, and by *Trichostrongylus* spp. and is considered to act in a way that protects the parasite (Sanderson and Ogilvie, 1971; Rothwell *et al.*, 1973). AChE has several *in vivo* effects that might aid in the worm's survival, for example it has been thought that it may act as a biochemical holdfast by reducing acetylcholine stimulated muscle contraction (Foster *et al.*, 1994). However, acetylcholine, the enzyme substrate also has many effects in immune response mechanisms such as the induction of rapid mucus secretion from goblet cells (Specian, 1980) and the induction of cellular immune responses (Strom *et al.*, 1974). A more plausible explanation for the high level of secreted AChE therefore is that it interferes with acetylcholine induced immune effector mechanisms directed against the parasite.

The amount of AChE secreted by *N. brasiliensis* increases through the course of an infection (Sanderson and Ogilvie, 1971). The isoform profile of the enzyme also changes with the amount of isotype "A" decreasing and being replaced by upregulation of isotypes "B" and "C" (Edwards *et al.*, 1971; Blackburn and Selkirk, 1992). These changes in AChE expression pattern correlate with parasite survival, suggesting that an immune response is generated against the enzyme and the worms are damaged and expelled by this developing immune response (Ogilvie and Jones, 1971). Any remaining "adapted" worms show high levels of the "A" isoform as well as "B" and "C" isoforms (Edwards *et al.*, 1971). This suggests that the "A" isoform is important in worm protection and that adapted worms have an increased capacity for its production in the face of immune pressure.

The changes in enzyme output are certainly influenced by the host immune status, such that an immune host will generate such changes more rapidly (Sanderson *et al.*, 1972; 1976). Infection with *N. brasiliensis* or *Trichostrongylus sp.* leads to the production of anti-AChE antibodies by the host (Rothwell *et al.*, 1973; Jones and Ogilvie, 1972). This response appears to account for the change in enzyme profile and consequent effects on survival of the worms described above (Edwards *et al.*, 1971; Jones and Ogilvie, 1972). These antibodies also indicate that the parasite protein is immunogenic.

A mechanism by which *N. brasiliensis* infection was resolved by the worms first being damaged by antibody and then removed by a second immune process was suggested by Ogilvie and Jones (1971). The observed immune response against AChE and the consequent changes in the AChE expression profile by *N. brasiliensis* might provide support for this hypothesis, and suggest that AChE is at least one target of the primary antibody response. The proposed removal of damaged worms by changes in mucus secretion (Ishsikawa *et al.*, 1994) would also fit this hypothesis. Mucus secretion from goblet cells may be stimulated by acetylcholine (Specian, 1980), worm expression of AChE may interfere with this process, hence protecting the worms from expulsion. An antibody response against AChE would therefore remove the interference and allow worm expulsion by the mucus secretion effector mechanism. Such a response would be the aim of vaccinating with recombinant AChE.

Previous attempts have been made at vaccination against *Trichostrongylus sp.* with AChE. In the Guinea pig / *T. colubriformis* model no significant level of protection was achieved (Rothwell and Merritt, 1975). Protection using ES protein extracts enriched for AChE was achieved in a Guinea pig model of *D. viviparus* infection but protection did not extend to challenged calves, this study being conducted with recombinant enzyme protein (McKeand *et al.*, 1995a; Matthews *et al.*, 2001). In a mixed nematode infection in sheep of *T. colubriformis, H. contortus* and *Cooperia oncophora* however, vaccination with *T. colubriformis* AChE led to a 31% reduction in total worm burden (Griffiths and Pritchard, 1994). The authors described these results as promising. This project will further investigate the use of recombinant AChE as a candidate vaccine.

N. brasiliensis AChE has previously been characterised, cloned and expressed as a recombinant protein (Blackburn and Selkirk, 1992; Hussein *et al.*, 1999). Recombinant AChE has been kindly made available for the planned vaccination trials by Dr M Selkirk of Imperial College, London.

1.10.7 Vaccine delivery

Previous vaccination trials have relied mainly on the use of systemic vaccination with recombinant or native parasite antigens (Gregg and Dineen, 1978; Emery *et al.*, 1993). The immune response to systemic vaccination is characterised by increases in serum immunoglobulin levels and specifically in IgG. It is likely that the immune responses generated in this way are not the most appropriate in protection against GI nematode infection, which may be modulated by local humoral and cellular responses.

Mucosal tissues are believed to share a common immune response. Stimulation of immune responses in the mucosal tissues of the gut may, therefore, be achievable through vaccination of other mucosal surfaces such as the nasal mucosa (Davis, 2001). In recent years interest has grown in the use of such alternative routes of vaccination. Methods for nasal vaccination have been developed for rodents and have been demonstrated against the trematode parasite *Schistosoma mansoni* (Akhiani, 1997) and also against GI nematode infection with *Ascaris suum* (Tsuji *et al.*, 2001) and *T. spiralis* (McGuire *et al.*, 2002). In these trials, in which the adjuvant cholera toxin was co-administered with the antigen, a significant immune response, polarised towards a Th-2 response, was generated. The nematode trials also achieved significant levels of protection associated with the local expression of IgG1 and IgA antibody responses both systemically and in the gut mucosa (Tsuji *et al.*, 2001); (McGuire *et al.*, 2002). Responses characterised as Th-2 and involving IgG1 and IgA also correlate with protection in the *N. brasiliensis* infection (Jarrett and Bazin, 1977). As the stimulation of local antibody responses demonstrated a protective effect in the above vaccination systems, it might, therefore, also be appropriate to stimulate such responses to achieve protection against *N. brasiliensis*. A further evaluation of nasal vaccination and its efficacy with recombinant antigens against intestinal nematodes is a main aim of the project.

1.11 *Nippostrongylus brasiliensis* as a model infection

N. brasiliensis has been used extensively as a model for GI nematode infection and immunity. There are a number of factors, besides its suitability for laboratory maintenance and abundance of reagents and techniques for use with the rat that suggest it might be a suitable model for *Trichostrongylus* infection.

Vaccination against helminth parasites has been investigated using a number of rodent model systems. These include *S.mansoni* (reviewed; McLaren, 1989), *A. suum* (Tsuji *et al.*, 2001) and *T. spiralis* (Goyal and Wakelin, 1993) in mice, *D. viviparus* (McKeand *et al.*, 1995a; McKeand *et al.*, 1995b) and *T. colubriformis* (Rothwell and Love, 1974; Rothwell and Merritt, 1975) in Guinea pigs and *N. brasiliensis* in both mice (Rhalem *et al.*, 1988a; Rhalem *et al.*, 1988b) and rats (Poulain *et al.*, 1976a; Murray *et al.*, 1979). In most of these studies parasite homogenates or purified parasite proteins have been delivered as antigens using a variety of formulations and routes of immunisation. Only more recently have studies conducted in these rodent vaccination models begun to utilise recombinant peptides and proteins (Tsuji *et al.*, 2001; McGuire *et al.*, 2002; Tsuji *et al.*, 2003).

Studies into vaccination against *N. brasiliensis* infection in both rats and mice have indicated that a high degree of protection can be generated by vaccinating with worm homogenate (Murray *et al.*, 1979) or ES material (Poulain *et al.*, 1976a; Rhalem *et al.*, 1988a; 1988b). Two of the ES components conferring protection in mice were partially purified and determined as 14 and 43 kDa proteins (Rhalem *et al.*, 1988b). In the rat host the highest degree of protection was afforded by immunisation with worm homogenates via the intra-peritoneal route using a *Bordetella pertussis* adjuvant (Murray *et al.*, 1979). An ES antigen was demonstrated to be more effective than homogenate when delivery was via the oral route (Poulain *et al.*, 1976a). The present study, however, is the first to propose the use of single recombinant antigen vaccines in this system, as a model for recombinant protein vaccination against GI nematode infection in ruminants.

The trickle infection regime with *N. brasiliensis* yields a stable, chronic infection, more similar to the natural infection and similar to the acquisition of ruminant nematodes on pasture (Jenkins and Phillipson, 1972). This chronic

infection is the basis of the suggested model system, with the modulation of this infection the aim of vaccination. A chronic infection in a rodent model is likely to be more informative than the more usually studied single challenge situation where the immune response is already rapidly excluding the worms, such as in the Guinea pig / *T. colubriformis* model. The trickle infection with *N. brasiliensis* has not to date been tested as a vaccination model. An evaluation of its potential by comparing the kinetics of single and trickle challenges following immunisation is one aim of the project.

The lifecycle and infective niche of *Trichostrongylus sp.* and the L4 and adult stages of *N. brasiliensis*, as well as pathology of infection and the immune responses invoked are very similar. Both infections may also provoke spontaneous expulsion and immunity to re-infection. Similar mechanisms of immunity may therefore exist between or be effectively provoked against these two nematode infections (summarised Table 1.2).

As far as has been established to date similar antigens, including SOD and AChE, which are of particular interest, are released by gut dwelling stages of both *Trichostrongylus sp.* and *N. brasiliensis*. Both of these enzymes are implicated as having a role in the ability of the parasite to evade the immune response of the host and therefore are obvious and rational targets for vaccination.

To examine the different systems of immunisation, systemic versus mucosal, a mucosal vaccination scheme is required. Nasal vaccination systems have been used effectively in a *S. mansoni* model (Akhiani, 1997) and GI nematode infections in mice (Tsuji *et al.*, 2001; McGuire *et al.*, 2002). A similar system may be an applicable to a model of a small intestinal species in rats.

1.12 Aims and objectives:

1.12.1 The aim of the project

To evaluate the *N. brasiliensis* / rat system as a vaccination model for intestinal nematodes, primarily small intestinal species of genus *Trichostrongylus*, by testing putative protective antigens and comparing delivery systems.

1.12.2 The objectives of the project

1. Establishing an appropriate infection regime for the challenge of vaccinated animals

2. Development of methods with which to monitor the immune response to infection and immunisation.

3. Production and characterisation of recombinant antigens. Enzymically active recombinant AChE is available through a collaboration with Prof M Selkirk, Imperial College, London. The cloning and expression of SOD is an early objective of this project.

4. An evaluation of AChE and SOD as vaccine candidates.

5. A comparison of the immunogenicity of systemic verses mucosal delivery systems.

Chapter 2 – Materials and Methods

2.1 General parasitological techniques

2.1.1 *Nippostrongylus brasiliensis* infections

Infections were carried out as described previously (Ogilvie and Jones, 1971). Unless otherwise indicated all donor or trial animals used were groups of male or female Wistar rats 2-3 months old. Rats were either anaesthetised with O_2/NO_2/halothane or suitably restrained and inoculated with *N. brasiliensis* L3 larvae prepared as described below. Immunisation with L3 was carried out in 0.2 - 0.5 ml phosphate buffered saline (PBS, Appendix 1), subcutaneously above the hind leg.

Donor animals, immunised with approximately 2000 L3, were culled at 7 days post infection (dpi) near the anticipated peak of egg output and worm burden. Faeces were collected for larval cultures from 6 dpi and adult worms were collected from the small intestine at 7 dpi.

2.1.2 Recovery of *Nippostrongylus brasiliensis* larvae

Infective stage (L3) larvae were obtained by faecal culture from infected donor animals. Faecal pellets from donor rats at 6 - 7 dpi were moistened, broken up and mixed with activated charcoal granules (Sigma, C-2889). This mixture was cultured at 20°C, on a Petri dish lined with damp filter paper (Whatman No. 1, 70 mm, 1001 070). L3 larvae were present on the plates from four days following culture.

Larvae were collected from cultures on the day they were required for use using a modified Baermann apparatus. This consisted of a funnel with a tap on the outflow and containing a 1 mm mesh, lined with "non-fluffy" paper towelling. The apparatus was filled with warm water (approximately 30°C) until the paper was covered and the larvae from the cultutre plates were gently washed in by filling the plates with water and pouring them into the apparatus. Larvae migrated downwards

and collected in the outflow of the funnel after approximately 45 min. Following collection larvae were washed 3 times in 10 volumes PBS with penicillin to a final concentration of 500 IU/ml and streptomycin to 500 mg/ml. Larvae for mRNA or protein extraction were dried by blotting with a corner of paper towel and snap frozen in liquid N_2 or stored at -80°C respectively. L3 in several aliquots were counted under a low power microscope x 5 to give a mean count and number of L3 per unit volume adjusted to give required doses.

2.1.3 Recovery of *Nippostrongylus brasiliensis* adults

Adult *N. brasiliensis* were recovered from the small intestine of donor rats at 7 dpi, around the peak of infection. Rats were euthanised, using CO_2, and the small intestine removed. The small intestine contents were removed and the small intestine slit open and added to the collected contents.

The collected material was then placed at the centre of a cotton gauze square (Millipledge, DG09075). This was suspended by the corners from a cocktail stick into a 100 ml beaker of PBS at 37°C. Adult worms migrated from the intestinal material through the gauze and were collected in the bottom of the beaker.

Collected adults were allowed to settle and then washed in a similar manner to the L3 described above. Adult worms were snap frozen in liquid N_2 for mRNA extraction, or stored at –80°C for protein fractionation until required.

2.1.4 Faecal egg counts

Faecal egg counts were carried out daily from 5 dpi onwards to the termination of infection, as the primary measure of infection. The method used for collection and counting was a modified saline floatation method (Jackson, 1974).

Fresh faecal samples (approximately 1 g) were weighed and broken up in water at 10 ml per gram. A 10 ml sample was then removed, strained to remove large debris and centrifuged at 100g for 2 min. The supernatant was removed and the

pellet re-suspended in a saturated saline (NaCl) solution. The suspension was centrifuged again at 100g for 2 min. The eggs float to the surface in the saturated saline and were removed into a 2 ml cuvette. Eggs were then counted manually under a x 40 microscope and results expressed as eggs/g faeces.

2.1.5 Adult worm counts

Adult worms were recovered as described above, and counted manually under a low power (x3) microscope on a watch glass marked with a grid. For accurate total worm counts, small intestine contents were collected and the gut slit open along its length. This material was then incubated at 37°C in pre-warmed (37°C) PBS for 2 h after which the mucosa easily detaches from the inner surface of the gut and was stripped and added to the contents. Formalin (Sigma, F1558-7) was then added to 2%, to preserve the material and the worms were counted under a low power microscope.

2.2 General molecular biology techniques

2.2.1 RNA extraction

Total RNA extraction from adult worms and L3 larvae

Adult worms stored in liquid N_2 (approximately 0.5 - 1 g) were crushed, using a sterile mortar and pestle pre-chilled to –70°C, with 5 ml of denaturing buffer containing the chaotropic agent guanidine isothiocyanate and the reducing agent β-mercaptoethanol (Stratagene mRNA Isolation kit, 200347), until finely powdered. The mix was then thawed to room temperature, aliquoted into 1.5 ml tubes and the proteins extracted with 0.5 ml phenol / chloroform / isoamyl alcohol (Sigma, P-3803). The aqueous phase was removed to fresh tubes and RNA precipitated using 0.5 ml isopropanol. Extracted RNA was stored under isopropanol at -20°C or reconstituted in RNase free water and used immediately for cDNA synthesis.

Total RNA was extracted from L3 using the Nucleospin RNA extraction kit (Machery-Nagel, 740955.20). The procedure was as described in the kit protocol. Briefly, 10 to 30 mg of larvae (snap frozen in liquid N_2) were ground in liquid N_2 in a pre-chilled (-70°C) sterile mortar and pestle with frozen extraction buffer. The mixture was thawed and spin filtered in a microfuge to remove protein and debris. The lysate was then loaded onto a Nucleospin RNA column. Following a desalting wash, DNase was added directly to the column and incubated for 15 min at room temperature to remove contaminating DNA. The column was then washed twice with ethanol and RNA eluted in 60 μl RNase free water.

RNA extraction from rat tissues

mRNA was isolated from rat spleen, mucosa and mesentery samples collected at various time points through infection, using the Stratagene mRNA Isolation kit (Stratagene, 200347) as per the manufacturers instructions.

Approximately 0.5 g of rat tissue, snap frozen and stored in liquid LN_2, was crushed in 5 ml RNA extraction buffer. Following this, elution buffer (10 ml) was added and the mix centrifuged for 30 min at 11,000 g to remove proteins. The supernatant was added to 5 ml oligo dT cellulose and the mRNA allowed to bind for 15 min. The cellulose was then centrifuged at 700 g for 3 min and the supernatant removed. The pellet was re-suspended in 5 ml high salt buffer, re-pelleted and the supernatant removed, this wash being repeated twice in high salt buffer and once in 5 ml low salt buffer. The cellulose was then packed into a push column and the mRNA eluted in 3 aliquots of 400 µl elution buffer at 65°C.

2.2.2 Reverse transcription (cDNA synthesis)

First strand cDNAs were reverse transcribed from total or mRNA using cDNA Cycle Kit (Invitrogen, 45-0014) or Superscript First Strand Synthesis System (Invitrogen, 12371-019) as described in the manufacturers protocols. Approximately 0.5 µg RNA was reverse transcribed using the Moloney Monkey Virus or Superscript reverse transcriptase. Transcription was primed from oligo-dT primers, which hybridise to the poly A^+ tail of the mRNA. Reactions were carried out in kit buffers and thermal cycling was performed on a Perkin-Elmer 2400 Geneamp thermal cycler using the manufacturers recommended conditions. Following transcription, the first strand cDNA was removed from the reaction mixture by addition of an equal volume of phenol /chloroform /isoamyl alcohol (Sigma, P-3803) and vortexing briefly. The tube was then centrifuged at 11,000 g for 5 min and the aqueous phase containing the cDNA removed to a fresh tube and stored at -20°C until further use.

2.2.3 Amplification of DNA sequences by polymerase chain reaction

Specific DNA sequences were amplified from cDNA templates by PCR using oligonucleotide primers of around 20 to 30 bases designed to bind specifically with the target sequence. In general, PCR reactions were carried out using the Expand Long Template PCR System (Roche, 1681834). Reactions were performed in 25 µl reaction mixtures containing:

10X buffer ($MgCl_2$ 22.5 mM); 0.4 mM dNTPs; 1 µM Primer 1; 1 µM Primer 2; 1.75 u Taq polymerase; 50-100 ng of DNA template.

Thermal cycling was carried out on a Perkin Elmer 2400 Geneamp thermal cycler. In general the cycle conditions were 4 min at 95°C to denature the DNA, followed by 25 - 40 extension cycles of 10 s at 95°C, 1 min at 50 - 60°C and 2 min at 68°C. A final extension phase was 7 min at 72°C.

2.2.4 Separation and visualisation of nucleic acids by agarose gel electrophoresis

Nucleic acids (RNA, cDNA and PCR products) were separated and visualised on agarose gels containing ethidium bromide (EtBr). EtBr intercalates into the DNA helix and fluoresces under UV light. Gels containing 0.8–2% (w/v) agarose in Tris / acetic acid / EDTA buffer (TAE. Appendix 1) and EtBr at 0.1 µg/ml were poured in gel trays (Pharmacia Biotech, 18-2400-02) with the required sample well size. Samples were loaded into wells on the gel in an equal volume of loading buffer (Appendix 1) under TAE buffer. Electrophoresis was then carried out at 80 V for approximately 30 min in TAE buffer. Nucleic acids in the gels could then be visualised using a UV light box and photographed.

2.2.5 Determination of nucleic acid concentration using a spectrophotometer

Nucleic acid concentrations were routinely estimated in solution using a spectrophotometer (Beckman, DU 650). The optical density (OD) at 260 and 280 nm of samples and blanks were measured and nucleic acid concentration and purity with regard to contaminating proteins was estimated. OD 260 was used as a measure of nucleic acid concentration with a relevant correction factor (RNA = 0.04 μg/μl/OD unit, DNA=0.05 μg/μl/OD unit). OD 260/280 was used as a measure of purity with a ratio of close to 1.8 being considered to contain relatively few impurities.

2.2.6 Polymerase chain reaction product purification

PCR products were prepared for sequencing by removing PCR reagents and contaminants using a Qiaquick PCR prep kit (Qiagen, 28704) as per instructions. PCR products were separated on agarose gels and bands excised under UV. The excised agarose containing the PCR product was dissolved in sodium iodide (NaI) solution at 55°C and the DNA then bound to silica in a spin flow column. This was washed with 70% ethanol wash solution and the DNA eluted in sterile distilled water. PCR products for cloning into bacterial vectors were cleaned by Geneclean 3 kit (Bio 101/Q-biogene, 1001-400) following the manufacturers protocol, similar to the above. This protocol appeared to preserve the adenine (A) nucleotide overhangs at the ends of the PCR products more efficiently than the Qiagen kit.

2.2.7 Cloning of polymerase chain reaction products in pGEM-T bacterial vector

Prior to sequencing, PCR products were cloned in pGEM-T vector system 1 (Promega, A3600). This vector is a high copy number, bacterial, plasmid vector. PCR products can be cloned directly into the vector for propagation and sequencing as it contains thymine (T) overhangs on its free ends.

Purified PCR product (approximately 25 ng) was added to pre-cut vector (approximately 50 ng) in a 3:1 molar ratio in a ligation reaction comprising; 2x buffer, 3 U DNA ligase, made up to 10 μl with sterile distilled water. Following incubation for 16 h at 4°C, a sample of the ligation solution (5 μl) was then transformed into 50 μl of JM109 competent cells (Stratagene, 200235) by heat shock at 42°C for 45 s. Following 1 h outgrowth in 500 μl SOC medium (Appendix 1), 200 μl of transformed cells were plated on Luria / agar (Appendix 1) plates with ampicillin at 50 μg/ml and grown overnight at 37°C. Colonies were picked into 10 ml Luria broth with ampicillin at 50 μg/ml and incubated at 37°C overnight.

2.2.8 Recovery of plasmid DNA

Plasmid DNA was recovered from 1.5 ml of overnight cultures using a Promega Wizard miniprep kit (Promega, A7100) as per instructions. Cells were disrupted in kit lysis buffer and the debris and proteins removed by centrifugation at 11,000 g for 30 min. DNA was bound to silica in spin columns and washed x2 with ethanol based wash buffer. Plasmid DNA was then eluted in 100 μl sterile distilled water (dH_2O). Dilutions of 1 in 100 of the eluted DNA were screened for positive inserts by PCR using specific primers.

2.2.9 Sequencing of polymerase chain reaction products

PCR products were sequenced in house at Moredun Functional Genomics Unit using a PE Biosystems 377 DNA sequencer and Big Dye terminator chemistry version 1.1 (Applied Biosystems, 433:7450). This is a chain termination sequencing method, and was performed according to manufacturer instructions.

In brief, cloned PCR products were used as template DNA in PCR reactions, primed with vector, or insert, sequence specific oligonucleotide primers. A percentage of the nucleotides in the reaction mix were di-deoxy nucleotides (d-dNTPs). These terminate the polymerisation of the DNA molecule at the point at

which they are included by the polymerase. The d-dNTPs were included at a concentration such that DNA molecules were produced at lengths differing by one nucleotide, with a d-dNTP represented at each position in the DNA sequence. The Big Dye system labels each d-dNTP species, adenine, thymidine, guanidine and cytidine (A, T, C and G) with a different coloured dye. The reaction products were then separated on an acrylamide gel that resolves a one-nucleotide difference. Dyed d-dNTPs were scanned as they passed by a laser, allowing the chain terminating species to be determined for each length of DNA molecule. The order of passing bases corresponds to the sequence of the template DNA.

Sequencing output and data were analysed using Chromas 1.43 (Technelysium Pty Ltd.) and DNA Star (DNA Star Inc.) software packages. The sequence of the 5' and 3' end of the SOD gene and the full length SOD coding sequence produced by primers P1 and P5 were determined in this manner. Further analysis of gene and protein sequences was performed using software packages available from EBI and NCBI websites. These included homology searches using BLAST, alignment with homologous sequences using Clustal-W and protein motif searching using Signal-P and Prosite websites.

2.3 General protein techniques

2.3.1 Adult worm somatic extracts

Somatic extracts (Adult S1) were prepared from adult *N. brasiliensis,* which had been stored at -80°C. Worms were thawed on ice and homogenised in ice cold PBS using a 1 ml hand held glass homogeniser (Jencons, England). Homogenates were then cleared by centrifugation in a microfuge at 11,000 g for 10 min and stored at -80°C until required.

2.3.2 L3 larvae somatic extracts

Larval somatic extracts (L3 S1) were prepared from L3 larvae cultured and prepared as described earlier and stored at -80°C. Worms were thawed on ice and homogenised in ice cold PBS, in a 2 ml vial containing glass beads (Hybaid, 60-100a-5G02), using a ribolyser (Bio 101/Thermo savant, Fastprep FP120). This method was used, as larval stages of nematode parasites are small and resistant to breakdown in a standard glass homogeniser. L3 were given 6x pulses of 30 s and cooled on ice for 30 min between each pulse. When the L3 were judged to be sufficiently disrupted, by examination of supernatant under dissecting microscope, the resultant homogenate was cleared by centrifugation at 11,000 g for 10 min and the supernatant retained. The pellet was re-extracted with a further 2 ml ice cold PBS, re-centrifuged and the supernatant added to the previous sample. Homogenates were stored at -80°C until required.

2.3.3 Determination of protein concentration

Determination of protein concentration using a spectrophotometer

For general use protein concentration in solution was measured using a spectrophotometer (Beckman, DU650). OD at 260 and 280 nm was measured against a PBS blank and the protein concentration was estimated from the OD 280 nm – OD 260 nm with a correction factor applied (1.55xOD 280 - 0.77xOD 260).

Determination of protein concentration by Pierce assay

When more accurate protein concentrations were required, protein concentration was determined by Pierce BCA protein assay (Pierce, 23225) using the kit protocol. This assay is based on the colour change of bicinchonic acid produced by the reduction of copper ions. Unknown samples and bovine serum albumin (BSA, Sigma, A-7906) standards were incubated in BCA working reagent (containing sodium carbonate, sodium bicarbonate, bicinchonic acid and sodium tartrate, 0.1 M sodium hydroxide and 0.1 % (w/v) copper sulphate) on a microtitre plate for 30 min at 37°C allowing colour formation. OD at 562 nm was then measured and the protein concentration determined by comparison of unknowns against the plotted standard curve.

2.3.4 Separation of proteins by poly acrylamide gel electrophoresis

Sodium dodecyl sulphate poly-acrylamide gels

Proteins were routinely fractionated using sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE), under either reducing or non-reducing conditions. Mini gels of approximately 9 x 6 cm and 1-2 mm thick were poured using the Mini Protean gel system (BioRad). These comprised a 10-15% acrylamide

resolving gel in separating buffer (Appendix 1), over-laid with a 4% acrylamide stacking gel in stacking buffer (Appendix 1). Ammonium persulphate (APS, Sigma, A-3678, 0.0005%) and TEMED (Sigma, T-9281; 0.001%) were added to gel mixes to facilitate polymerisation. Gels were typically cast with 10 x 30 μl wells. Proteins, S1 extracts, column or cell fractions, were loaded in either reducing or non-reducing loading buffer (Appendix 1). Reducing samples were incubated at >95°C for 5 min and then placed on ice prior to loading. Reducing samples in this manner allows accurate sizing of protein bands following fractionation. Protein size markers (Invitrogen, LC5677) were also loaded in adjacent lanes to determine the size of separated proteins.

Proteins were fractionated using the Mini Protean gel tanks at 200 V for approximately 45 min in Laemmli electrode buffer (Appendix 1).

Native poly-acrylamide gel electrophoresis

Protein gels that required fractionated proteins to remain in their native unreduced conformation were performed under native PAGE gel conditions. Gels were poured with 10-15% acrylamide in Tris / glycine buffer (Appendix 1) as above but with no SDS. Loading buffer was Tris / glycerol / bromphenol blue (Appendix 1) without SDS or β-mercaptoethanol and samples were not boiled prior to loading. Similarly, the electrode buffer used was Tris / glycine without SDS (Appendix 1) and for gels that were to be stained for enzyme activity this was pre-chilled on ice to 4°C before use. Fractionation conditions were 200 V for 45 min as before and when gels were to be stained for enzyme activity, the apparatus was kept on ice for this period.

Staining poly-acrylamide gels for protein with Coomassie blue

Following fractionation gels were removed from the glass plates and stained to visualise the proteins. Staining was generally carried out using Coomassie blue stain (Appendix 1). Gels were stained in 20 ml stain for 30 min and then washed for

3 x 45 min in methanol / acetic acid destain (Appendix 1) to remove the background staining. Gels were then photographed or dried onto 3 mm chromatography paper (Whatman, 3030917) as a permanent record.

2.3.5 Detection of proteins by immuno-blot (Western blotting)

Transfer of proteins to PVDF membranes

Proteins separated by SDS-PAGE under reducing or non-reducing conditions were transferred to PVDF membranes prior to detection of proteins with specific antibody. Gels were removed from the glass plates and rinsed in dH_2O. Transfer was carried out using a "semi-dry" electroblot method. Following electrophoretic separation the gel was removed from the apparatus and washed briefly in dH_2O. The gel was then placed on the anode of the electroblotter (Milliblot Graphite Electroblotter 1, Millipore, MBBDGE001), on top of a layer of PVDF membrane (Immobilon P™, Millipore, IVH0010) that had been activated by immersion in methanol and rinsed in dH_2O. This overlaid two pieces of 3 mm chromatography paper, the lower soaked in 0.3 M Tris (Appendix 1) and the other in 0.025 M Tris (Appendix 1). The gel was then covered by a third layer of chromatography paper soaked in 0.025 mM Tris / glycine (Appendix 1) and the cathode of the blotter. Transfer was carried out at 150 mA of current for 45 min following which layers were removed and the membrane rinsed in distilled water.

Visualisation of protein on membranes by reversible staining with Ponceau Red

Proteins were visualised on membrane blots by staining with 0.1% Ponceau S solution (Sigma, P-3504) for 5 min. Separated protein bands stain red allowing lanes to be distinguished. At this point lanes could be separated for permanent staining with Coomassie or probing with specific antibody. Membranes were then rinsed

clear of the stain in Tris buffered saline with Tween-20 (TBST, Appendix 1) for 30 min.

Probing blots with specific antibody

Detection of individual protein bands on a gel was accomplished by probing with specific antibody. Blots to be probed were incubated overnight in 10% dried milk powder (Marvel, Premier Brands) in TBST to block non-specific binding. Following blocking, membranes were washed for 15 min x3 in TBST. The membrane was then incubated in specific anti-sera at the appropriate dilution in TBST for 2 h and washed as above. Specific antibody was then detected on the blot by incubating with secondary antibody, conjugated to horseradish peroxidase (HRP). Blots were incubated with secondary antibody HRP conjugates at appropriate dilutions in 10% Marvel / TBST for 2 h. Blots were then visualised by incubating the membranes in 3, 3' diaminobenzidine substrate (Sigma Fast DAB, D-4418) and washed thoroughly in dH_2O.

Visualisation of blots using ECL+Plus

For more sensitive detection of specific antibody binding the ECL+Plus (Amersham Pharmacia Biotech, RPN 2132) was used. This detection system is based on the production of chemi-luminescence by a reaction catalysed by the conjugated HRP. The luminescence produced is then detected by exposure of autoradiography film. ECL was used as per manufacturers protocol. The membrane was covered with the ECL+Plus buffer containing Lumigen PS-3 acridan substrate and incubated for 5 min. The membrane was then drained and wrapped in clingfilm. Exposure of autoradiography film was carried out in X-ray film cassette for the required time based on initial results.

2.4 Enzyme activity assays

2.4.1 Superoxide dismutase activity assay

SOD activity was detected and measured using an assay based on the oxidation of iodo-nitro-tetrazolium violet (INT) by the xanthine / xanthine oxidase system (Jones and Suttle, 1981). SOD activity in the assay was indicated by the inhibition of the purple colour formation, produced as the INT is oxidised.

Solutions containing, samples of protein (12.5 μl) were added to wells of a 96 well, flat bottomed microtitre plate in 100 μl, 0.25 mM carbonate buffer at pH 10.2 (Appendix 1) with 0.1 mM xanthine (Sigma, X-0626), 0.025 mM INT (Sigma, I-8377). The reaction was initiated by the addition of 0.01 U of xanthine oxidase (Boehringer Mannheim, 110434) and colour formation allowed to progress for 30 min. The OD at 492 nm was then determined using a microplate reader (Bio-tek Instruments Inc., ELX808iu). OD is used as a measure of colour and therefore substrate formation. SOD activity could be detected by the inhibition of colour formation and quantified against a series of SOD standards of known concentration (Sigma, S-2515). Activity is defined as 1 U producing 50% inhibition of colour formation.

2.4.2 Acetylcholinesterase activity assay

AChE activity was determined using an assay adapted from the method of Ellman et al (1961), as described in Blackburn and Selkirk (1992). Samples of protein in solution (20 μl) were added to wells of a microtitre plate containing 200 μl 0.1 M phosphate buffer, pH 7 (Appendix 1) with 0.2 mM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB, Sigma, D-8130) and 1.5 mM acetylthiocholine iodide (Sigma, A-5751). The colour forming reaction was allowed to progress for 5 min, following which the OD at 414 nm was determined as a measure of substrate formation.

2.4.3 Staining poly-acrylamide gels for superoxide dismutase activity

Proteins were fractionated by gel electrophoresis and stained for SOD activity (Beauchamp and Fridovich, 1971). Proteins separated on 15% non-reducing SDS-PAGE, as described above, were removed from the glass plates and washed x4 in 2 % Triton X-100 (Sigma, X-100) for 15 min. This wash removes the SDS and allows the protein to refold to its original conformation.

SOD activity was then visualised by incubating gels in a solution containing 2.45 mM nitro-blue tetrazolium (Sigma, N-6639) in dH_2O for 20 min. This was followed by incubation in a solution containing 28 mM TEMED (Sigma, T-9281), 28 μM riboflavin (Sigma, R-4500) in 0.033 M phosphate buffer pH 7 (Appendix 1) for 15 min. Gels were then developed by exposure to light, on a light box, for 5-15 min. This causes superoxide ion (O_2^-) production and reduction of the nitro-blue tetrazolium with the formation of a blue colour in the gel. SOD activity inhibits this reduction and was indicated by clear zones on a blue background.

2.4.4 Staining native poly-acrylamide gels for acetylcholinesterase activity

AChE activity was visualised on native page gels using the method of Karnovsky and Roots (1964). Proteins were separated on 10% PAGE gels as described previously, using native conditions and ice-cold buffer, with the electrophoresis performed on ice. Following fractionation, the gels were removed from the glass plates and incubated in a solution containing 0.1 M phosphate at pH 6.5 (Appendix 1) with 2.6 mM acetylthiocholine iodide. The following were then added to final concentration; 5 mM sodium citrate, 3 mM copper sulphate, 0.5 mM potassium ferricyanide and incubated for >30 min. AChE activity was visualised as brown bands in the gel. Following several rinses in dH_2O, gels could be photographed or dried.

2.4.5 Staining native poly-acrylamide gels for esterase activity

Gels were stained for non-specific esterase activity using the method of Grunder et al (1965). Separation of proteins using native PAGE gels was carried out as described above using ice cold buffers and with the gel tank cooled in ice during electrophoresis. Gels were then removed and stained in 5 mg/ml fast blue RR salt (Sigma, F-0500) in PBS with 1/20 volume 2% naphthyl acetate (Sigma, N-8505) in acetone. Dark brown bands of colour developed after around 5 min and the development was then stopped by several washes in distilled water. Gels could then be photographed or dried onto 3 mm chromatography paper.

2.5 Establishment of the infection model

2.5.1 Investigation of single challenge and re-infection

Groups of 3 Wistar rats were infected with 2,000 L3 as described above and the infection was allowed to progress through to expulsion of the worms and termination of infection. FEC were carried out from day 5 as the primary measure of infection and the number of adult worms were also counted around the peak of infection.

Rat tissue samples; spleen, mesentery and intestinal mucosa were collected at time points 0, 1, 7, 11 and 14 days during the infection. Tissues from an uninfected control group of three rats were taken on day 0. These tissues were snap frozen in liquid N_2 to preserve the RNA for development of a reverse transcription PCR method to monitor host cytokine expression.

2.5.2 Investigation of varying challenge doses

Animals used in the investigation of challenge dose were infected, as detailed above, with varying numbers of L3 from 25 to 500. Some animals were infected, at weekly intervals, with 25 L3 or 3x weekly with 5 L3. FEC were performed as the primary measure of these infections.

2.6 Molecular cloning and expression of *Nippostrongylus brasiliensis* superoxide dismutase

2.6.1 SMART cDNA synthesis

Total RNA from *N. brasiliensis* adults was used to produce cDNA using the SMART cDNA Library Construction Kit (Clontech, PT 3001/PR92334, K1051-1). This was carried out according to the SMART cDNA synthesis by Long Distance Polymerase Chain Reaction (LD-PCR) protocol in the user manual.

In brief, this method is a reverse transcription that produces a first strand cDNA, with specific oligonucleotide sequences incorporated at the 5' and 3' end of each strand. The cDNA can then be amplified non-specifically by PCR using primers directed at these incorporated sequences. The product is a SMART cDNA, with the SMART III oligonucleotide incorporated at 5' end and the CDS III oligonucleotide at the 3' end (Table 2.1).

2.6.2 Amplification of *Nippostrongylus brasiliensis* superoxide dismutase coding DNA by polymerase chain reaction

Gene specific primers for SOD (Table 2.1) were designed to regions of the gene sequence flanking the SOD active site domains, which is highly conserved between nematode species. Some sequence data were also available from previously determined partial *N. brasiliensis* SOD gene sequences (Young, 1996).

Primer binding sites are outlined in Figure 2.1, below. The 5' end gene product was amplified by priming with the P 1 primer against the antisense P 4 primer. PCR amplification of the SOD 3' end gene product was accomplished by priming with P 2 sense primer towards the 3' poly-A tail and the incorporated CDS III oligonucleotide in the SMART cDNA template. Internal SOD products were amplified as controls using P 2, sense and P 3 and P 4 antisense primers.

The products of these reactions were the 5' and 3' halves of the gene sequence and internal SOD products respectively. The products were sequenced and the data used to design a gene specific primer for the 3' end of the coding sequence, P 5.

Primer	Binding site	Sequence
SMART III	5' end cDNA	AAG CAG TGG TAT CAA CGC AGA GTG GCC ATT ATG GCC GGG
P 1	5' end SOD gene	ATG AGC AAC CGA GCA GTG GCC GTG
P 2	Conserved region	CTG TAT CTC TTG TGG ACC TCA CTT TAA
P 3	Conserved region	CGA CTC CTC CTT CTT CTC ACC GAC TAC
P 4	Conserved region	GTG ATT TCG AAA TGA GCA ACA CCA CTG GC
P 5	3' end SOD gene	TCA CTG GGG AGC AGC GAT GGC GAT CAC TCC
CDS III	3' end of cDNA	ATT CTA GAG GCC GAG GCG GCC GAC ATG-d(T)30N_{-1}N

Table 2.1. The oligonucleotide primers used in the amplification of SOD gene specific products. Primers specific for the conserved region and cDNA ends were used to amplify products which were subsequently sequences and aligned to provide a contiguous full length gene sequence. P1; start codon underlined. CDS III; N_{-1} =A, G or C, N= A, T, G or C.

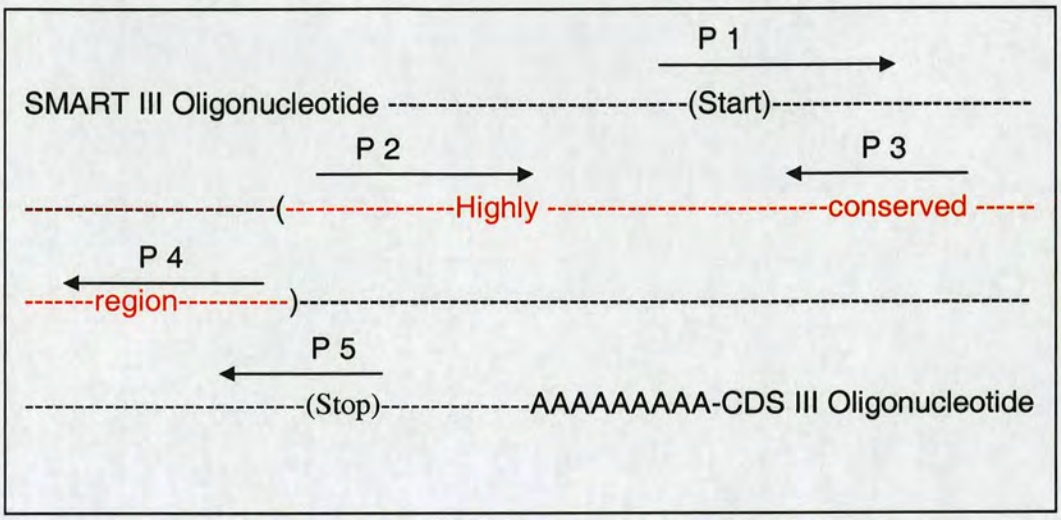


Figure 2.1. Line diagram showing the outline of the SOD cDNA with primer sites, P 1 - P 5, Start and Stop codon, conserved region and the attached SMART oligonucleotides. Primers specific for the conserved region and cDNA ends were used to amplify products which were subsequently sequences and aligned to provide a contiguous full length gene sequence.

2.6.3 Cloning of superoxide dismutase cDNA into pET22b+ expression vector

A SOD full-length cDNA was amplified by PCR using P1 and P5 primers with incorporated *Bam*H1 and *Eco*R1 restriction enzyme sites respectively (Table 2.2). These restriction sites were chosen to allow cloning in frame into pET22b+ bacterial expression vector (Novagen, 69744-3). This vector was chosen as it had previously been used to express an enzymically active *H. contortus* SODc (Liddell and Knox, 1998).

P 1 *Bam*H1	CGT A**G↓G ATC C**GA TGA GCA ACG GAG CAG TG
P 5 *Eco*R1	GTC TA**G↓ AAT TC**A GTC ACT GGG GAG CAG C

Table 2.2. The oligonucleotide primers used in the amplification and subsequent cloning of the full length SOD gene for *in vitro* expression in *E. coli*. (Restriction sites shown in bold).

Around 1 μg of SOD product and 800 ng of pET22b+ were prepared for ligation by restriction digest with *Bam*H1 and *Eco*R1 restriction enzymes (Roche, 567604 and 200310). Restriction digest reactions (20 μl), comprising of 2 μl 10x buffer B; 10 U *Eco*R1; 10 U *Bam*H1; sterile dH_2O to 20 μl, were incubated at 37°C for 3 h. The DNA was then removed from the other reagents using Geneclean 3 kit following the protocol described in section 2.2.6. Around 50 ng of prepared SOD product was ligated into 50 ng of prepared pET22b+ in 10x ligation buffer with 10 U T4 DNA ligase (Roche, 481220) made up to 10 μl with distilled water and incubated at 4°C overnight.

2.6.4 Transformation into competent *Escherichia coli* strains

Escherichia coli JM109 competent cells were used to propagate the SOD/pET22b+ construct prior to expression in *E. coli* BL21 cells, as these are more efficient transformants. Transformations were carried out as described previously and plasmid DNA recovered by miniprep. Plasmid DNA positive for the SOD insert was identified by PCR screening with SOD specific internal primers as described above.

Around 35 ng of positive plasmid were transformed into 50 μl BL21 competent cells (Stratagene, 200133) by heat shock at 42°C for 20 s. The BL21 cell type is suitable for expressing recombinant proteins. A control of pET22b+ vector containing no insert was also transformed. Following 1 h outgrowth in 500 μl SOC medium, 200 μl of transformed cells were plated on Luria / agar plates with ampicillin at 50 μg/ml and grown overnight at 37°C.

2.6.5 Induction of expression

Colonies containing the SOD/pET22b+ construct and pET22b+ alone control in BL21 cells were picked into 10 ml Luria broth (Appendix 1) with ampicillin at 50 μg/ml and incubated at 37°C overnight. Sub-samples of these cultures (1 ml) were

then inoculated into 10 ml aliquots of Luria broth containing ampicillin (Stratagene, 300021-61) at 50 μg/ml. These were incubated with shaking at 37°C for around 3 h until the OD at 600 nm reached over 0.6. Isopropylthio-β-D-galactoside (IPTG, Bioline, BIO 37036) was then added to one culture of SOD/pET22b+ construct and one of pET22b+ alone to a concentration of 1 mM. IPTG induces the cells to express proteins by binding to and blocking a transcription repressor site. Cultures were incubated for a further 2 h and aliquots removed to examine the expressed proteins.

2.6.6 Preparation and analysis of protein fractions

Samples, 1.5 ml, of the induced and non-induced cultures were pelleted at full speed in a microcentrifuge for 5 min and the supernatant retained as the media fraction.

Periplasmic, soluble and insoluble cell fractions were prepared to test for localisation of the expressed protein. Periplasmic fractions were prepared by incubating cells from one 1.5 ml aliquot in chloroform at room temperature for 15 min then adding 75 μl of 10 mM Tris / HCl pH 8. This was then centrifuged at 11,000 g for 15 min and the aqueous phase retained as the periplasmic fraction.

Soluble and insoluble cell fractions were prepared from cells by lysis in 100 μl of 50 mM Tris pH 8.5 / 2 mM EDTA with lysozyme at 100 μg/ml and Triton X-100 at 0.1%. $MgCl_2$ was added to 8 mM, DNase 1 (Boehringer, 104159) and RNase A (Boehringer, 109169) to 10 μg/ml. The lysate was pelleted at full speed in a microcentrifuge for 10 min with the supernatant forming the soluble cell fraction and the pellet, re-suspended in 100 μl, 50 mM Tris pH 8.5 / 2 mM EDTA forming the insoluble fraction.

Samples of 10 μl of these fractions were separated on 15% SDS PAGE gels, and stained using Coomassie blue.

2.6.7 Purification of recombinant superoxide dismutase protein by ion exchange chromatography

Recombinant SOD was recovered from the culture media or supernatant of overnight cultures by ion exchange chromatography in a manner similar to that described for *H. contortus* SOD (Liddell and Knox, 1998). Cell debris was removed from 500 ml overnight cultures induced with IPTG by centrifugation at 1700 g for 30 min at 4°C. The supernatant was decanted into 50 ml aliquots and stored at -20°C until required. Aliquots (100 ml) of supernatant were concentrated using centrifugal filters (Centricon, Ym-10) to <10 ml prior to ion exchange separation. Media proteins were then fractionated on a Mono-Q sepharose ion exchange column using a Pharmacia LKB (LCC-500/P5-500) FPLC chromatography system. Salts and other media contaminants were first removed from the sample by filtration through a Sephadex G25 column (Pharmacia) with the sample proteins being eluted in 10 ml of 10 mM Tris/HCl pH 7.5 (Appendix 1). Samples were then loaded onto the Mono Q column, which bound most proteins in the column matrix. Unbound material was eluted with 10 mM Tris/HCl and the bound proteins were eluted using a salt gradient of 0-0.3 M NaCl over 30 min. Eluted proteins were collected in 2 ml fractions which were analysed by SDS-PAGE, Western blot and for SOD activity. This showed that a fraction enriched for the recombinant SOD and of relatively high purity eluted at around 0.07 M NaCl.

2.7 Detection of immunological markers of infection

2.7.1 Detection of immune markers by enzyme linked immunosorbent assay

Extraction of soluble antibody from mucosa samples

Mucosa samples were obtained by scraping the mucosal surface of ~10 cm of the anterior small intestine with a glass microscope slide. Samples were homogenised in 2 ml ice cold PBS, on ice, using a 5 ml, glass homogeniser (Jencons) to solublise the antibody in the tissue. Following extraction, samples were centifuged at 11,000 g for 10 min to remove cell debris and normalised to 10 mg/ml protein.

Development of enzyme linked immunosorbent assay to detect antibody isotypes from sera and mucosa

An enzyme linked immunosorbent assay (ELISA) method was developed for the detection of the antibody isotypes total IgG, IgG1, IgG2a, IgA and IgE in sera and mucosal tissue from primary challenge infected animals and controls.

Microlon (high binding) ELISA plates (Greiner/Bio-one, 655061) were coated with 1 μg/ml of adult *N. brasiliensis* S1 antigen, in 50 μl / well bicarbonate buffer pH 9.6 (Appendix 1) at 4°C overnight. Plates were then washed 3x with TBST (Appendix 1) and incubated overnight at 4°C with 200 μl / well 10% Marvel / TBST to block non-specific binding. Plates were again washed x6 with TBST.

Sera samples and mucosal homogenates (primary antibody) were used in dilution series from 1 in 20 to 1 in 20,480 in PBS and neat to 1 in 1,024 in PBS respectively. Primary antibody was added in 50 μl / well PBS and incubated for 1 h at room temperature following which plates were washed x6 as above.

Individual, isotype-specific secondary antibodies (shown in Table 2.3) were then added (one per plate) at a range of dilutions. Secondary antibodies were diluted in TBST (50 μl / well) and incubated on the plates for 1 h at room temperature.

Following washing x6 as above, the horse radish peroxidase (HRP) conjugated anti-IgG secondary antibody could then be detected by the addition of 100 μl / well Sigma Fast–OPD (Sigma, P-9187) and the colour allowed to develop for around 15 min before stopping with 2.5 M H_2SO_4.

The other antibody isotypes were detected by incubation with HRP conjugated, anti-mouse immunoglobulin antibody (see Table 2.3), for a further 1 h before detection with Sigma Fast–OPD in the same manner. Following colour development, OD values for each well were read at 450 nm using a plate reader. OD values for each dilution of secondary antibody with infected and uninfected control samples were then plotted on a graph to determine the most appropriate working dilution of each.

Antibody	Type	Supplier	Cat. No.
Anti-rat IgG	HRP conjugated	Sigma	R-9037
Anti-rat IgG1		Serotec	MCA194
Anti-rat IgG2a		Sigma	R-0761
Anti-rat IgA		Sigma	R-0636
Anti-rat IgE		Serotec	MCA193
Anti-mouse Ig	HRP conjugated	Dako	P0260

Table 2.3. The sources of monoclonal antibodies used in the detection of rat immunoglobulin isotypes IgG, IgG1, IgG2a, IgA and IgE by ELISA. All antibodies were raised in mice and detected using the Anti-mouse Ig HRP conjugated antibody, with the exception of the anti-IgG antibody, which was HRP conjugated.

Detection of antigen specific serum and mucosal antibody using enzyme linked immuno sorbent assay

Antigen specific IgG, IgG1, IgG2a, IgA and IgE were detected and quantified in sera and mucosal samples from vaccinated animals by ELISA in a manner similar to that described above. ELISA plates were coated with 1 μg/ml of antigen (SOD or AChE) in bicarbonate buffer pH 9.6 (Appendix 1) overnight. Plates were then washed and blocked overnight with 10 % Marvel / TBST (Appendix 1) and again washed x6 with TBST.

Primary antibody (sera or mucosal homogenates prepared as above) was then added in dilution series in PBS and incubated for 1 h following which plates were washed x6 as above. Isotype-specific secondary antibody was then added at the appropriate dilution and incubated for a further 1 h. Following washing x6 as above the HRP conjugated anti-IgG was then detected as above and the other isotypes detected with anti-mouse HRP conjugated antibody. Following colour development OD values for each well were read at 450 nm using a plate reader. Specific antibody levels for vaccinated or infected animals were then calculated as a titre against specific antibody levels in dilutions of control sera (1 in 20) or mucosal homogenate (10 mg/ml).

Quantification of rat mast cell protease II levels by enzyme linked immuno sorbent assay

Rat mast cell protease II (RMCP II) levels were detected and quantified from sera and mucosal tissues by ELISA in the manner of Miller et al (1983b), using a commercially available kit (Moredun Scientific Limited, RMCP II ELISA). Assays were carried out according to manufacturer's instructions. ELISA plates were coated with 2 μg/ml anti-RMCP II monoclonal antibody in bicarbonate buffer pH 9.6 (Appendix 1) at 4°C overnight. Following 6x washes with PBS with Tween-20 (PBST, Appendix 1) plates were blocked at 37°C for 30 min with 4% Bovine serum albumin in PBST. Plates were washed again x3 and standards in series from 0.5 – 12

ng/ml of RMCP II in 4% BSA / PBST were added in triplicate. Unknown samples (sera or mucosal homogenates at 10 mg/ml) were then plated in dilution series 1 in 10 to 1 in 10,000 and incubated at 37°C for 30 min. Following a further wash x6, plates were incubated for 1 h with 50 μl/well conjugated antibody solution at 37°C. Plates were once again washed x6 before addition of 50 μl/well substrate. Colour formation was allowed to progress for around 15 min before stopping with 0.25 M H_2SO_4. OD values for each well were then read at 450 nm on a microplate reader and RMCPII levels calculated by comparison against a plotted standard curve.

2.7.2 Detection of rat cytokine expression by reverse transcription - polymerase chain reaction

cDNAs prepared from rat tissues were used as template in a PCR experiment to detect cytokine gene mRNA transcription. Oligonucleotide primers (Table 2.4) were obtained for a subset of rat Th-1 and Th-2 cytokines to show the relative Th-1/Th-2 bias of the immune response during *N. brasiliensis* infection. Primer sequences were obtained from Matsuda *et al.* (1995). β-Actin primers were included as a constituitively expressed control to allow comparison between experiments.

Cytokine	Primer sequence
IL-2 F	CAT GTA CAG CAT GCA GCT CGC ATC C
R	CCA CCA CAG TTG CTG GCT CAT CAT C
IL-3 F	TGC CTT GGA GAT TTT GGT GAA GCT CCC
R	CAG GTC CTT AAG ATG GAT CAC GTA GA
IL-10 F	TGC CAA GCC TTG TCA GAA ATG ATC AAG
R	GTA TCC AGA GGG TCT TCA GCT TCT CTC
IFN-γ F	ATG AGT GCT ACA CGC CGC GTC TTG G
R	GAG TTC ATT GAC AGC TTT GTG CTG G
IL-4 F	ACC TTG CTG TCA CCC TGT TCT GC
R	GTT GTG AGC GTG GAC TCA TTC ACG
IL-5 F	TGC TTC TGT GCT TGA ACG TTC TAA C
R	TTC TCT TTT TGT CCG TCA ATG TAT TTC
β-Actin F	AGA AGA GCT ATG AGC TGC CTG ACG
R	CTT CTG CAT CCT GTC AGC GAT GC

Table 2.4. Oligonucleotide primers specific for rat cytokine and a constituitively expressed control (β-Actin) cDNA sequence (Matsuda *et al.*, 1995). The primers were used in PCR with template cDNA isolated from the tissues of Wistar rats infected with 2000 *Nippostrongylus brasiliensis* L3 larvae to detect cytokine transcription.

2.8 Immunisation of rats with recombinant antigens

2.8.1 Trial 1 - Subcutaneous immunisation with recombinant superoxide dismutase and acetylcholinesterase

Experimental design

The immunogenicity and protective capacity of recombinant SOD and AChE was initially assessed using a systemic vaccination regime. Previous vaccination trials in ruminants have utilised systemic regimes (Smith, 1999), a comparison of these antigens with these previous trials was considered a useful starting point. The systemic regime chosen was subcutaneous (SC) vaccination. An adjuvant, Quil A, considered to boost both Th-1 and Th-2 systemic immune responses, was used in conjunction with the antigens to increase the immune response. Challenge doses for the experiment were low level single challenge doses, having been considered the most appropriate in previous investigations of challenge dose.

Immunisation regime

One group of rats was immunised with 10 μg SOD and one with AChE in 0.2 ml PBS with 10 μg of Quil A adjuvant (Superfos Biosector, Batch L77-194). Another group of seven, control, rats were not immunised and one further control group immunised with 10 μg Quil A alone. All animals were housed in cages of 2 or 3 animals. The rats were initially immunised on day 0 of the experiment and boosts of the same vaccination dose were given at weeks 4 and 8. All immunisations were performed subcutaneously above a hind limb while the rats were suitably restrained.

All groups were challenged at 2 w after the final boost (week 10) with 25 L3 larvae in 0.2 ml PBS injected subcutaneously above a hind limb as described previously. The infection was allowed to progress for 11 d and all animals culled for necropsy.

Experimental measurements

FEC were taken from day 5 – 11 post challenge as the primary measure of infection. Samples were collected from each cage and egg counts performed as described previously (Section 2.1). Results were expressed as eggs / g faeces for each cage giving 3 samples per group or as a mean of each group. Cumulative egg output through the course of infection was also calculated and compared between groups using analysis of variance (one way ANOVA) as a measure of the statistical significance of any differences.

Blood samples were collected prior to final boost by tail bleed (week 6). Sera from these samples was assayed for antigen (SOD / AChE) specific antibody, to confirm that an immune response had been stimulated by the immunising doses prior to challenge and to provide pre-challenge antibody levels. Antibody levels were assayed by ELISA as described previously (Section 2.7).

On necropsy, samples were taken for immunological and parasitological analyses. Blood (sera) and mucosal scrapings (harvested as described above) were collected for analysis of RMCP II and antibody levels by ELISA. The remaining small intestine and contents were retained for adult worm counts.

2.8.2 Trial 2 – Intra-peritoneal immunisation with acetylcholinesterase

Experimental design

Part 1- Intra-peritoneal immunisation with acetylcholinesterase

This experiment was carried out to test the immune response and the degree of protective immunity stimulated by an immunisation regime designed to produce mucosal Th-2 responses. Mucosal and Th-2 responses may be more appropriate than systemic response in the immune response to GI nematodes. The preferential targeting of such responses might therefore enhance protection. The previous systemic immunisation regime was repeated to confirm the results. In addition a

further group of seven rats was added. This group was immunised with AChE intra-peritoneally (IP), a route of immunisation which might enhance mucosal immune responses compared to systemic antigen delivery (Murray *et al.*, 1979). In this group Alhydrogel (aluminium hydroxide gel, Sigma, A-8222) was chosen as an adjuvant as it is considered to enhance Th-2 responses (Nicklas, 1992).

Part 2 – Immunisation of Sprague Dawley rats with superoxide dismutase

Based on the results of the previous systemic trial with SOD, this experiment was designed to test whether the Wistar strain of rat had failed to recognise the foreign SOD protein. Genetic restriction of major histocompatibility complex II (MHC II) may occur in rat strains, preventing immune recognition of antigens. The previous immunisation schedule was repeated with two groups, one immunised and one control, of Sprague-Dawley rats to test whether an immune response was invoked in this strain. This strain has been used in previous *N. brasiliensis* infection studies.

Immunisation regime

Part 1: A group of seven Wistar rats were immunised with 10 μg AChE with Quil A subcutaneously as before. Another group of seven Wistar rats was immunised by the IP route, with 10 μg of AChE with Alhydrogel (Sigma, A-8222) in 0.2 ml PBS. Prior to immunisation, AChE was absorbed to an equal volume of Alhydrogel for 2 h on ice. Absorption was measured at >60% by spectrophotometer. Doses were then diluted to the required volume in PBS. One further group of seven, control, Wistar rats was not immunised. Immunisations were given on day 0 followed by boosts at week 4 and 8 as before.

Part 2: One group of seven Wistar and one group of seven Sprague-Dawley rats were immunised with 10 μg of SOD with 10 μg Quil A in a SC regime as described

previously. A group of seven control Sprague-Dawley rats was not immunised. Immunisation and boosts were performed to the time schedule outlined above.

All groups were challenged 2 w after their final boost with 25 L3 larvae injected SC as before. The infection was allowed to progress for 11 d and the rats then culled for the collection of samples at necropsy.

Experimental measurements

Blood (sera) was collected by tail bleed one week prior to infection (week 9) for the measurement of pre-challenge specific antibody levels by ELISA. Post infection FEC were performed from day 5 as the primary measure of infection. At necropsy sera (blood) and mucosal scrapings were collected for measurement of immune markers and the small intestine retained for adult worm counts.

2.8.3 Trial 3 – Intra-nasal immunisation with AChE

Experimental design

Stimulation of the immune response at a mucosal site in the nasal passage is thought to elicit responses at other mucosal surfaces via the common mucosal immune system (Davis, 2001). Following the results of the previous experiments, this experiment was carried out to further test the specific targeting of immunisation to provoke immune responses at the gut mucosal surface. Responses at this site might provide a greater degree of protection from mucosally associated nematode parasites. Previous experimental groups immunised via the SC and IP routes were repeated to give replicate results. A further group was immunised via the intra-nasal (IN) route to assess the immune response and degree of protection afforded by this route.

Immunisation schedule

Groups of seven Wistar rats were immunised SC or IP with AChE as described above as well as an infection challenge control group, which was not immunised. A further group of seven Wistar rats was immunised with 10 μg of AChE via the nasal passage using 10 μg of cholera toxin β-subunit (CTB) as an adjuvant. AChE and CTB were administered in 50 μl of PBS to the external nares using a micropipette on days 0, 14 and 21 timed to coincide with the final boost of the other groups. All groups were challenged 2 w after their final boost with 25 L3 in 0.2 ml saline as previously described.

Experimental measurements

Sera were collected by tail bleed 1 w prior to challenge to monitor pre-challenge antibody levels by ELISA. FEC were measured from day 5 post infection as the primary measure of infection. Egg count data were analysed using a repeated measures model described in Section 2.8.4 below. At necropsy sera and mucosal scrapes were collected for analysis of immune markers and the small intestine retained for adult worm counts.

2.8.4 Statistical analysis of vaccination trial egg count data

Where indicated above any differences present in the cumulative egg counts between vaccinated and control groups were analysed by analysis of variance (one way ANOVA) to measure the statistical significance of the difference.

Egg count data from the vaccinated and control animals in Trial 3 was analysed using a repeated measures model, an advanced statistical test. The counts were analysed on a log scale and the repeated measures model was fitted in Genstat

7th Edition, using an autoregressive model for the correlation between counts from the same animal on successive days.

As one group of animals in Trial 3, vaccinated with AChE via the SC route, was the same as one of the experimental groups in Trial 1, the egg counts of control and AChE vaccinated animals from Trial 1 could also be included in the repeated measures model. It was valid to include these data as they represented a repeated experiment in this test. Inclusion of this data further increased the statistical power of the test.

Chapter 3 - Establishment of the infection model

3.1 Introduction

Single challenge infection with several thousand L3 larvae has so far been the most studied experimental infection regime using *N. brasiliensis* in rats (reviewed; Rothwell, 1989). In this regime the FEC begins to rise at around 5 DPI and peaks around 7 DPI with a corresponding peak in adult worm numbers in the small intestine (Ogilvie and Jones, 1971). At this time, moderate pathology and inflammation is apparent in the mucosa. Following this, the FEC and number of adult worms counted in the small intestine decline with the infection being resolved between 10-14 DPI when egg counts and worm numbers approach zero. Some authors report a threshold population that survive expulsion and maintain a chronic low-level infection after the bulk of the infecting dose has been expelled (Ogilvie and Jones, 1971).

In the present study, a single challenge with 2,000 L3 larvae was performed in order to confirm that the dynamics of infection were the same in the strain of rats used, namely Wistars. Parasitological and immunological markers of infection were measured during this acute single challenge infection. This experiment put future experimental challenges into the context of previous work carried out using this system (reviewed; Ogilvie and Jones, 1971; Miller, 1984).

Such a severe, acute infection may not be the most appropriate challenge for testing a vaccine. If the infecting dose were too high, the challenge might overwhelm any protective effect conferred by vaccination. A high infection dose might also stimulate an immediate protective response in primed hosts that would not be observed at a lower, more natural, level of infection.

Prior to vaccination trials therefore, experiments were also carried out to establish low level infection regimes, closer to infection levels that may be anticipated through natural exposure. These experiments involved infecting groups of rats with varying levels of challenge infection. FEC of these groups was monitored through the course of infection, as this was to be the main indicator used to monitor challenges to vaccinated groups.

Single dose challenge infections have to date been the most used regime for challenging vaccinated animals in ruminant trials (Knox, 2000; Knox and Smith, 2001). However, grazing livestock are usually exposed to infection by the continuous (trickle) ingestion of infective larvae from pasture over a prolonged period of time (Waller and Thomas, 1981; Dobson *et al.*, 1990). A trickle challenge regime with *N. brasiliensis* has previously been shown to yield a long lasting, chronic infection in rats. This infection is stable and refractory to the expulsion that has been observed in an acute challenge (Jenkins and Phillipson, 1972). This infection regime might be the most desirable challenge to vaccinated animals, being the closest to a natural acquisition of GI nematode infection. Trickle challenge infection was tested for potential use as a challenge infection regime for vaccinated animals in this study.

3.2 Results

3.2.1 Faecal egg count during acute single challenge with 2000 L3

A group of 15 rats was infected with 2,000 L3 per animal in the manner described in Chapter 2.1. The infection was allowed to progress for 12 days and FEC were performed daily. Egg counts were performed on a pooled sample collected from each cage of 3 rats giving a mean from 5 counts for each timepoint. The outcome of this analysis is summarised in Figure 3.1. Eggs were detectable in faeces from day 5 post infection peaking on day 7 at around 2,300 eggs per gram of faeces and then dropping rapidly such that eggs were undetectable in faeces by day 10. However, egg output was very variable at each time point as judged by the large standard error of the mean counts.

3.2.2 Adult worm count during acute single challenge

During the course of the infection, groups of 3 animals were culled for adult worm counts at regular time-points. The numbers of adult worms in the small intestine were counted by the method described in Chapter 2.1.3 and the results are shown in Figure 3.2. Worm numbers in the intestine increased from 3 DPI, peaking on day 7 and declining thereafter to trace numbers on day 11. At the peak of infection around 650 worms were present in the small intestine, around 32% of the infecting dose given. The variation in the worm counts was relatively small as shown by the low standard error.

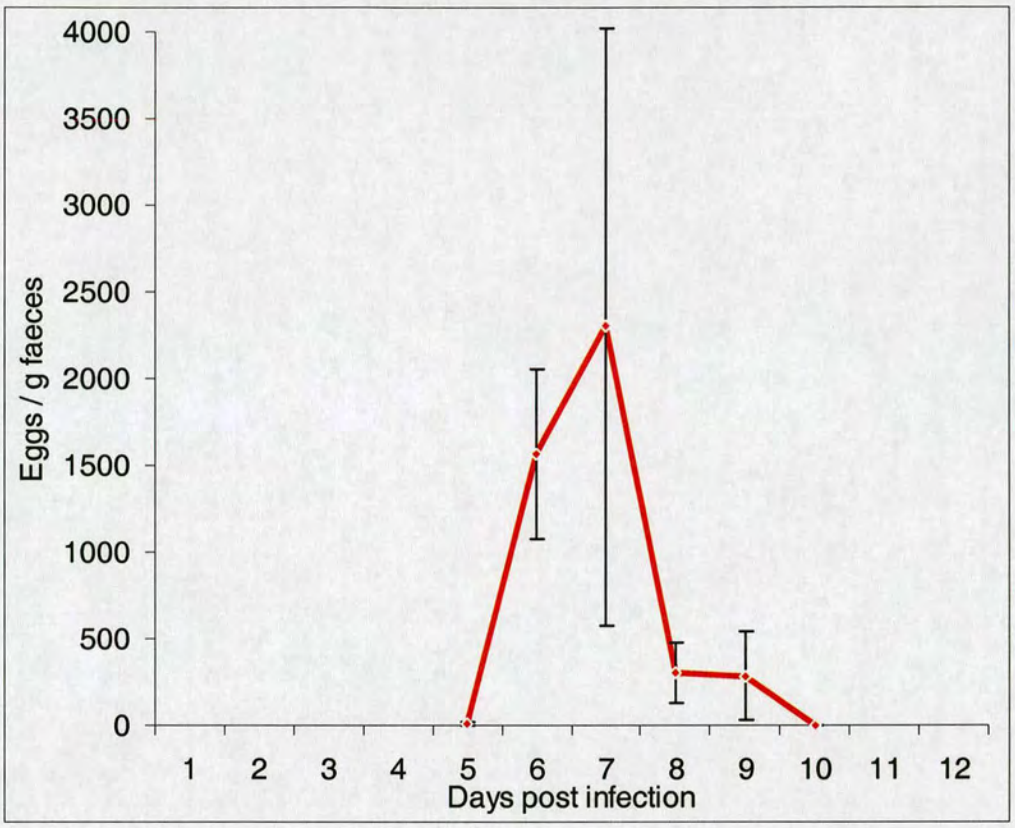


Figure 3.1. The mean faecal egg output over 10 days from a group of Wistar rats given a single challenge infection of 2,000 Nippostrongylus brasiliensis L3 larvae. The data represent the mean of egg counts performed on the pooled sample collected from each of 5 cages containing 3 rats, +/- the standard error. Egg count began to rise at 5 days after infection, peaked at 7 days and approached zero by 10 days.

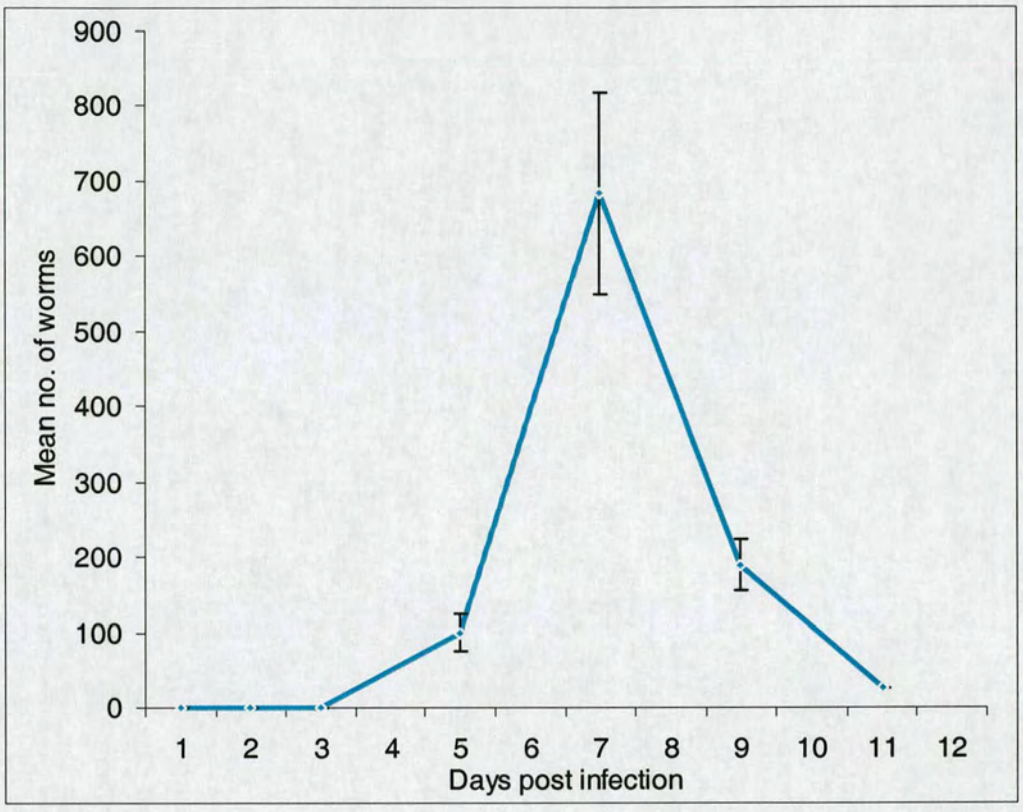


Figure 3.2. The worm burden over 11 days of a group of Wistar rats given a single challenge infection of 2,000 *Nippostrongylus brasiliensis* L3 larvae. Each point represents the mean of adult worm counts performed from three individual rats at each timepoint.

3.2.3 Serum immunological marker levels

Levels of parasite specific antibody and mast cell protease (RMCP II) were measured by ELISA in the sera of infected rats, as described in Chapter 2.7. Antibody levels were expressed as a titre compared to control serum antibody level and the results are displayed in Figure 3.3, panels A to E.

As shown in Figure 3.3, panel A, IgG titre began to rise around 9 DPI and increased until at least 23 DPI. The highest IgG titre, observed on day 23, was 3,000. The change in titre of IgG subtypes IgG1 and 2a are shown in panels B and C. The IgG1 titre showed an earlier peak than the IgG2a at 9 DPI, this correlating with the rejection of the infection. An increase in IgA and IgE was detectable earlier in infection, from day 7. IgA levels (Panel D) began to rise at day 7, peaking at around 700 on day 11 and were still elevated at day 14. IgE levels (Panel E) rose from 7 DPI and plateaued at around 900 on 11 DPI, this level being maintained until at least 23 DPI.

RMCP II levels (Panel F) were initially low, at around 300 ng/ml and showed a slight increase as early as the second day of infection. Between 7 and 11 DPI RMCP II levels increased, to around 2,500 ng/ml, before dropping again to baseline levels by 23 DPI.

The level of antibody was also measured by ELISA from the serum of a group of rats that were re-infected with 2,000 *N. brasiliensis* on day 23 post primary challenge and from a group of repeatedly challenged hyper-immune rats (data not shown). During the secondary challenge the antibody level appeared to peak 24 hours after challenge and the antibody titre was much higher than during primary challenge at around 7,000 for IgG, 6,000 for IgE and 3,000 for IgA. In hyper-immune animals the titres were also elevated, but not as highly as during the secondary challenge. There was also a comparatively lower proportion of IgA, and the IgG response appeared to be dominated by the IgG1 component. Levels of RMCP II were elevated in both cases with a very high level in the serum of the hyper-immune rats.

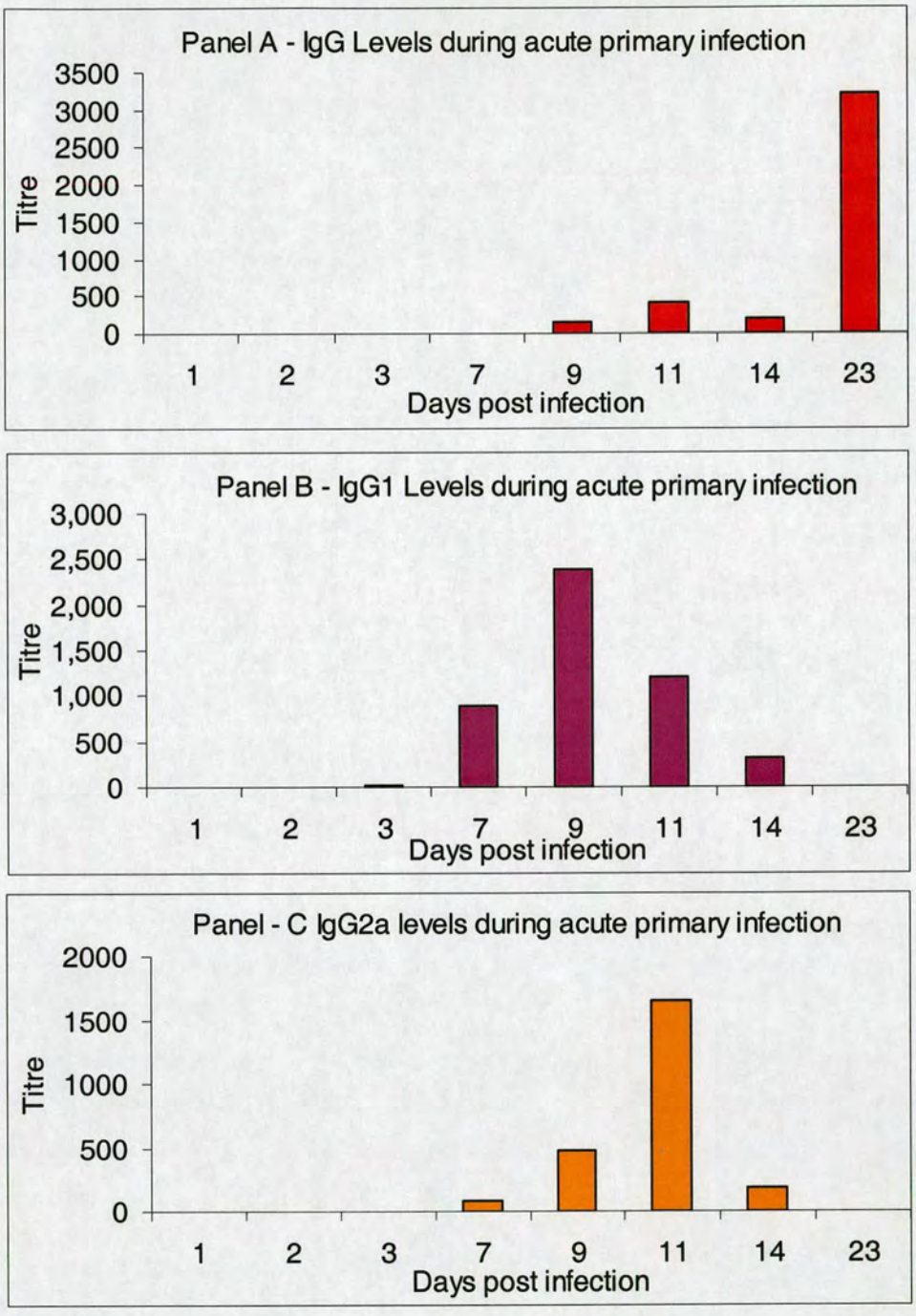
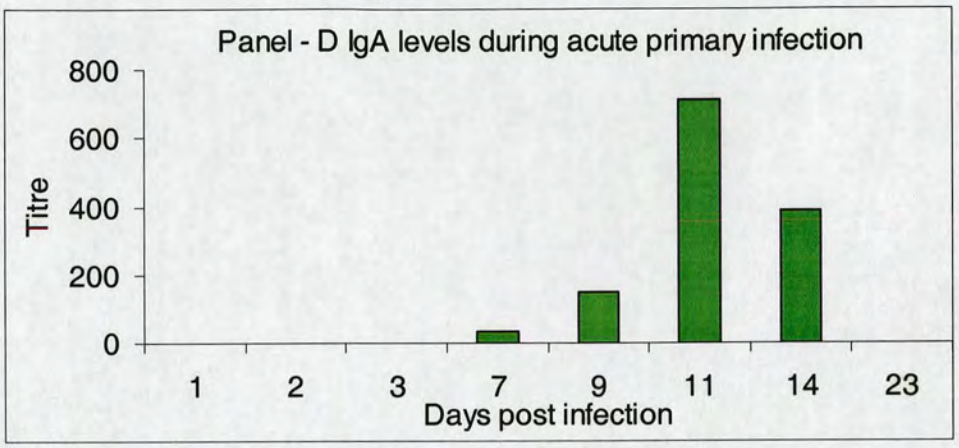
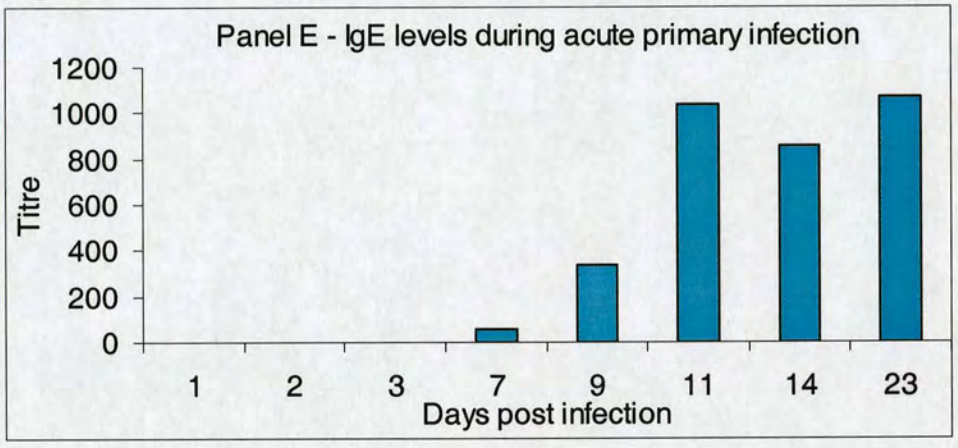


Figure 3.3 The parasite specific, serum IgG, IgG1, and IgG2a response of Wistar rats over 23 days following infection with 2000 *Nippostronglyus brasiliensis* L3 larvae. The antibody titres were measured by ELISA from a pooled serum sample of 3 individual rats at each timepoint and using soluble parasite (S1) extract as the capture antigen.





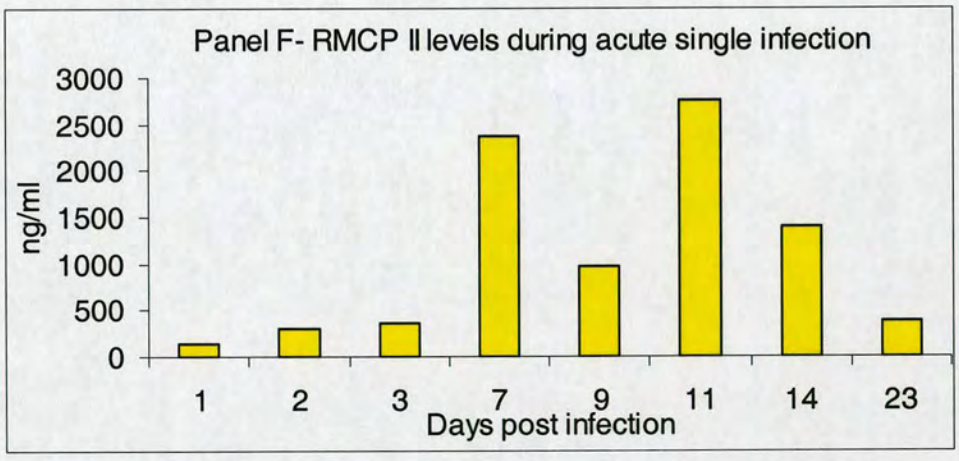


Figure 3.3 (continued) The parasite specific serum IgA and IgE response and the Mast Cell Protease (RMCP II) response of Wistar rats over 23 days following infection with 2000 *Nippostrongylus brasiliensis* L3 larvae. The antibody titres were measured by ELISA using soluble parasite (S1) extract as the capture antigen. The serum for these and the RMCP II ELISA were from a pooled sample of 3 individual rats at each timepoint.

3.2.4 Mucosal immunological marker levels

Levels of mucosal immune markers during the acute single infection were measured from samples of small intestine mucosa as described in Chapter 2.7. *N. brasiliensis* specific antibodies were detectable, although not at high levels. Titres for IgG, IgG1, IgG2a, IgA and IgE isotypes were calculated and are displayed in Figure 3.4, Panel A-E. The change in antibody isotype profile conformed to the general pattern seen for the serum antibody levels, but antibody was present at comparatively lower titres.

RMCP II levels, shown in Figure 3.4, Panel F, rose from baseline to peak levels around day 14 after infection, thereafter declining again to a lower level by 23 DPI. RMCP II levels in the mucosa were comparatively higher than those in serum, at 5,500 ng/mg soluble protein, on day 14, but again conformed to the general pattern seen.

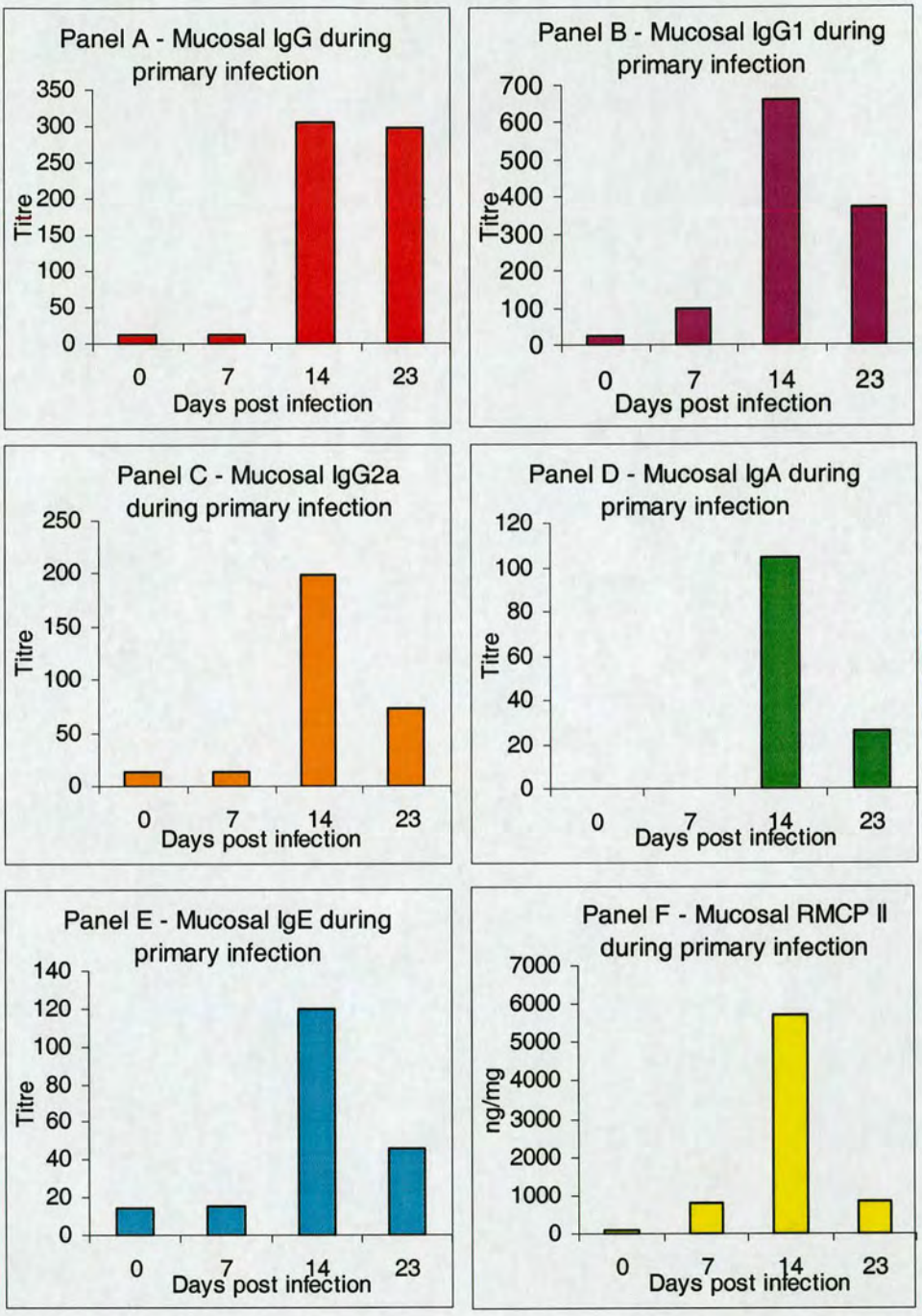


Figure 3.4 The parasite specific IgG, IgG1, IgG2a, IgA and IgE response and the RMCP II response in the intestinal mucosa of Wistar rats given a single challenge infection of 2,000 *Nippostrongylus brasiliensis* L3 larvae. The antibody titres were measured by ELISA using soluble parasite (S1) extract as the capture antigen. Antibody titre and RMCP II level were measured from a mucosal homogenate sample collected at each timepoint.

3.2.5 The effect of different levels of challenge

Single challenge infection

FEC results from groups of 6 animals infected with 25 and 50 L3 larvae are displayed in Figure 3.5, panels A and B respectively. A similar profile of egg output was seen at both infection levels with the egg count peaking at over 300 on 7 DPI and then declining gradually to 0 by 13 - 15 DPI. Somewhat surprisingly, the peak level of egg output was similar for both infection regimes whilst the FEC from the 25 L3 group was somewhat less variable than that of the 50 L3 group as demonstrated by the lower standard error.

Trickle challenge infection

Several trickle challenge regimes were tested to determine how appropriate such a regime might be to use as a challenge for vaccinated animals. FEC from animals infected with 25, 100 and 200 L3 each week peaked and fell to zero before 18 DPI in a manner similar to that seen for single challenge infection (data not shown). Continued weekly challenge of these animals did not produce a measurable egg output.

FEC results from a group of 6 animals infected with 5 L3 three times a week are shown in Figure 3.5, panel C. In this situation the egg count peaked and dipped several times before dropping to around zero at 14 DPI. During this phase the variability between counts (standard error) was relatively large. With continuing challenge, a stable, chronic egg output developed after around 21 DPI and persisted until at least day 35. The FEC during this phase was between 20-30 eggs / g faeces and the variability between counts was greatly reduced.

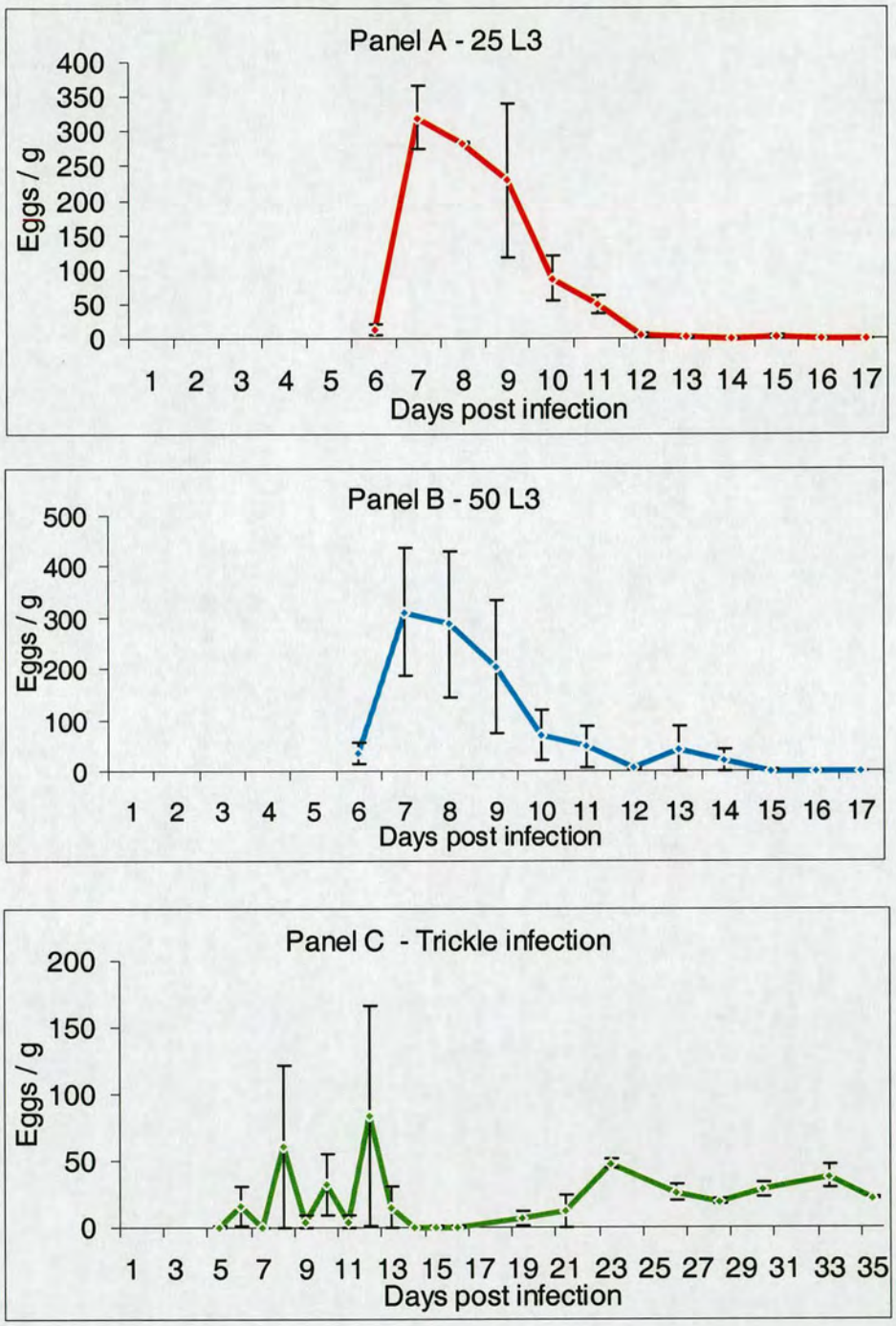


Figure 3.5. The mean faecal egg output over 17 days of a group of 6 rats infected with a low level single challenge of 25 Nippostrongylus brasiliensis L3 (Panel A) and 50 Nippostrongylus brasiliensis L3 (Panel B). Panel C indicates the mean faecal egg output over 35 days of a group of 6 rats given a trickle challenge infection with 5 Nippostrongylus brasiliensis L3 larvae three times a week.

3.3 Discussion

The results of the acute single challenge confirm the pattern of infection seen in previous studies (Ogilvie and Jones, 1971). The demonstrated parallel between FEC and worm count also confirms the use of FEC as an appropriate primary measure of infection. FEC can be taken from live animals and its use as the primary measure of infection thus allows smaller groups of animals to be used during vaccination trials compared to those required if worm counts were being used. In the latter, animals would have to be sacrificed at each time point.

The increases in levels of parasite-specific antibody during acute single challenge infection confirmed the results from previous studies showing an increase in specific IgG, IgA, and IgE in the serum (Jarrett and Bazin, 1977). Large increases in IgE in particular are associated with *N. brasiliensis* infection (Jones and Ogilvie, 1967; Ogilvie and Jones, 1971). Importantly, in this study, IgG1 and IgG2a correlated closely with the time of expulsion of the parasite. IgG1 and IgG2a are associated with Th-2 immune responses in rodents (Binder *et al.*, 1995) and IgG1 was demonstrated as the serum antibody isotype associated with the passive transfer of protection in this system (Jones *et al.*, 1970). Levels of parasite-specific antibody rose more rapidly and to even higher levels within 24 h in animals re-infected with 2,000 L3 on day 23 post primary infection and were similarly high in hyper-immune animals. The high IgG response of the hyper-immune rats had an increased component of IgG1.

Similar increases in specific antibody levels were detectable in the intestinal mucosa, an important site for protection against intestinal parasite infection (Miller, 1984). Levels of IgG were elevated from day 7 and peaked at day 14. This peak did not correspond with the peak in serum antibody titres indicating local production rather than leakage of antibody from the serum. Changes in IgA and IgG in the intestinal mucosa were similar to those reported previously (Poulain *et al.*, 1976b; Sinski and Holmes, 1977; Wedrychowicz *et al.*, 1983). These authors observed a peak of specific and total IgA in the intestine 12 DPI and IgG between 18 and 24 DPI. IgE was also seen to peak at 14 DPI in this study. IgE is not considered a secretory antibody isotype and this IgE might represent leakage of antibody from the

serum, or immune cell associated IgE both of which would be expected to peak at this time-point (Mayrhofer, 1977).

These data confirm that antibody may be involved in the resolution of primary and subsequent challenges with *N. brasiliensis*. Passive transfer experiments have previously implicated antibody in the rejection of *Nippostrongylus* infection, and this was associated with IgG1 (Miller, 1984; Jones *et al.*, 1970). A component of this IgG1 response was directed against a functional parasite protein, AChE, and the isotype profile of this enzyme changed through the course of infection, perhaps in response to this immune pressure (Sanderson and Ogilvie, 1971; Jones and Ogilvie, 1972). As this protein is thought to play a role in the persistence of the parasite such a specific antibody response may play a role in the expulsion of the worm (Jones and Ogilvie, 1972; Sanderson *et al.*, 1972). Raising an antibody response against functional parasite enzymes might therefore be an appropriate objective of vaccination.

RMCP II was measured from both the serum and mucosa during the acute single challenge infection. Higher levels were observed in the mucosa than in the serum. This is logical, as the mucosa is the source of circulating enzyme. Levels in both compartments showed the same pattern of elevation around the time of peak infection in the intestine and decline following expulsion and were similar to levels previously reported (Huntley *et al.*, 1993).

These results confirm that the infection behaved in a similar manner to that described in previous studies. This is true of both parasitological and immunological markers and allows any results from this study to be compared with those of previous work.

Experiments were also performed to define the most appropriate level of infection with which to challenge animals during forthcoming vaccination trials. The infection cycle during low-level (25 and 50 L3) single challenge infections was slightly more prolonged than the acute (2,000 L3) single challenge and the FEC did not drop as sharply. This lower level of infection might be more appropriate than an acute infection for vaccination challenge. It was decided to use an infection dose of 25 L3 during subsequent vaccination trials. Although similar levels of egg output

were seen during infections with 25 and 50 L3 the variation in egg counts, the standard error, was lowest for 25 L3.

An initial attempt to establish a cumulative, chronic infection by trickle challenge with weekly doses of 25 L3 was unsuccessful. This regime was devised to simulate the effect of infecting five times a week with 5 L3 in the manner of Jenkins and Philipson (Jenkins and Phillipson, 1972) whilst avoiding excessive trauma to animals. In this situation the animals expelled the primary infecting dose and remained immune to further challenge in a manner similar to that described previously for single challenge and re-infection (Ogilvie and Jones, 1971). Two other groups in this experiment, 100 and 200 L3 / week, had been intended to investigate any upper threshold level for the establishment of a cumulative, chronic infection. In this model such a threshold appears to be less than a single challenge with 25 L3.

In a subsequent experiment animals were infected with 5 L3 three times a week. A stable, chronic egg output was eventually established with this regime. This infection however produced a low FEC and was not cumulative in the manner of the infection produced by Jenkins and Phillipson (1972). It seems likely that the level of infection needed to produce such an infection is extremely critical lying between multiple doses of 5 and 25 L3. At the lower end of this scale expulsion is not provoked, but the number of worms does not accumulate, while at the upper, expulsion of the infection is elicited and the host becomes immune to further infecting doses.

Due to the failure of the tested infection regimes to produce the cumulative chronic infection demonstrated by Jenkins and Phillipson (1972), it was decided to use low-level single infection as a challenge dose in vaccination trials. The results of the experiment conducted by challenging with 3 x 5 L3 / week however indicate that it may be possible to reproduce such an infection and future work might provide a suitable regime for vaccination challenge in this model.

In summary, the experiments described in this chapter confirm that the infection behaves in a manner similar to that previously reported. The changes in egg output and worm numbers correlated closely allowing the former to be used as the primary indicator of challenge infection. Changes in specific antibody isotype and RMCP II levels also conform to the pattern described previously and correlated with

worm expulsion, indicating that they may be of importance in the resolution of infection. Finally, experiments with differing levels of infection have allowed the most appropriate challenge for the proposed vaccination studies to be decided.

Chapter 4 - Molecular cloning and expression of *Nippostrongylus brasiliensis* Cu / Zn superoxide dismutase

4.1 Introduction

As stated in the General Introduction (Chapter 1), Cu / Zn SOD is an enzyme involved in a pathway that facilitates the reduction of oxygen radicals (Henkle-Duhrsen and Kampkotter, 2001). These radicals are produced during normal cellular oxidative metabolism and cells contain several enzymes, including SOD, catalase and peroxidases, which remove them before they can cause damage (Chapter 1, Figure 1.4).

Protective immune responses to intestinal nematode infection are associated with eosinophil accumulation in the mucosa (Miller, 1984). These cells are capable of an extended respiratory burst resulting in the sustained release of large quantities of superoxide anion and other toxic products (Babior *et al.*, 1973). In *Nippostrongylus brasiliensis* infection, maximum eosinophil accumulation occurs at the time of, with degranulation occurring prior to, worm expulsion (Kelly and Ogilvie, 1972). *In vitro* studies have shown that eosinophils can accumulate around infective L3 larvae (Rainbird *et al.*, 1998; Shin *et al.*, 2001) and have also indicated that this cell type could kill the trapped larvae in the gut mucosa (Meeusen and Balic, 2000).

SOD is released by many parasitic nematode species (Knox and Jones, 1992) and can protect worms from host-mediated free radical release (Rhoads, 1983; Smith and Bryant, 1986; Kazura and Meshnick, 1984; Henkle-Duhrsen and Kampkotter, 2001). As such, it is a rational target for vaccination as an effective immune response against the enzyme might remove an important mechanism by which the worms protect themselves from damage and expulsion. A previous study on *H. contortus* suggested that Cu / Zn SOD may be effective as a vaccine candidate, giving a 20% reduction in worm burden (Liddell and Knox, 1998). One aim of this project was to further investigate the potential of this enzyme as a vaccine.

To accomplish this it was first necessary to clone and express *N. brasiliensis* Cu / Zn SOD *in vitro* for use as an antigen in vaccination studies. This was accomplished by a Rapid Amplification of cDNA Ends (RACE) PCR method that has previously been used to isolate *H. contortus* SOD (Liddell and Knox, 1998). This method selectively amplifies specific sequence using primers directed to highly-conserved or known regions of the gene sequence. The sequence ends were amplified from primers directed towards 5' or 3' conserved sequences, or oligonucleotide sequences incorporated into the 5' and 3' ends of the cDNA during synthesis.

Following amplification and confirmation that the reaction product did encode the target SOD by sequencing, gene primers were designed to allow cloning of the gene sequence in frame into a bacterial expression vector. Expression of the recombinant enzyme was induced and the enzyme subsequently purified from a bacterial culture. The purified enzyme was then examined for purity, solubility and activity, factors that may affect the efficacy as a vaccine.

SOD enzyme activity has previously been reported from somatic extracts of *N. brasiliensis* (Knox and Jones, 1992). In this study, expression of the native enzyme was examined in both the adult and L3 stages of the worm. The presence of the native enzyme in somatic extracts was demonstrated by Western blot. The expression of the *N. brasiliensis* Cu / Zn SOD encoding mRNAs was also examined by a semi-quantitative RT PCR to determine any gross differences in expression levels between adult and L3 stages.

4.2 Results

4.2.1 Isolation of *Nippostrongylus brasiliensis* Cu / Zn superoxide dismutase – encoding cDNA

Messenger RNA extraction from *Nippostrongylus brasiliensis* adults

mRNA was extracted from adult *N. brasiliensis* as described in Chapter 2.2.1. The amount of recovered RNA was 130 μg as determined from the ratio of OD_{260}: OD_{280} nm. A 10 μl sample of this RNA was separated in 0.8% agarose, visualised with EtBr staining and photographed under UV for analysis (Figure 4.1, panel A). A smear of RNA was clearly visible extending over a broad size range, confirming the integrity of the mRNA.

cDNA synthesis from *Nippostrongylus brasiliensis* adult RNA

Around 0.5 μg of total RNA was used to produce SMART cDNA as described in Chapter 2.6.1. Following first strand synthesis and amplification by Long Distance PCR, 10 μl of cDNA were visualised using 0.8 % agarose gel electrophoresis and EtBr staining (Figure 4.1, panel B). The cDNA shows a bright smear between 0.1 and 4 kb indicating a high yield of cDNA across the molecular weight range.

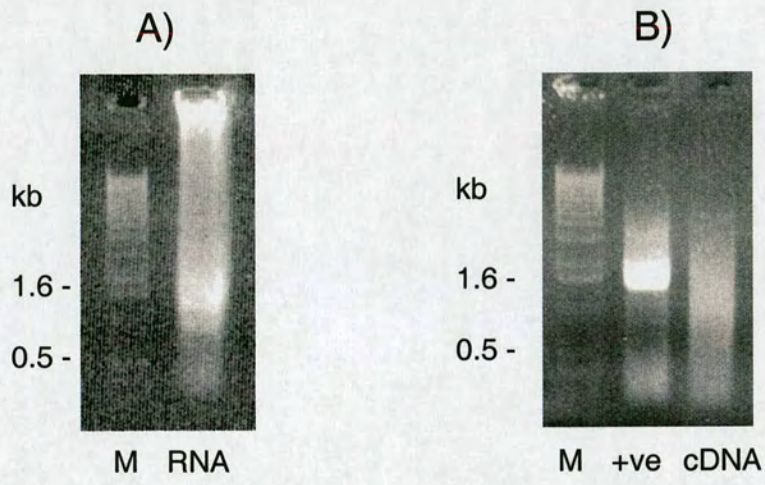


Figure 4.1. Panel A) mRNA extracted from adult *Nippostrongylus brasiliensis*. The smear extending through a broad size range and lack of low molecular weight degradation products indicates the integrity of the RNA. Panel B) is the corresponding cDNA, reverse-transcribed using a SMART cDNA kit (+ve = cDNA synthesis positive control, a prominent band of the expected size is visible at 1.6 kb; M= molecular weight marker).

4.2.2 SOD PCR products

SOD gene-specific sequences were amplified from the *N. brasiliensis* adult cDNA as described in Chapter 2.6.2. Primers were designed to a region of the gene with high homology between nematode species and against the integrated oligonucleotides of the SMART cDNA 5' and 3' ends. The primer sequences are shown in Chapter 2.6.2 and the expected product sizes are indicated in Table 4.1. The products from PCR amplification after gel electrophoresis in 0.8% agarose gels are shown in Figure 4.2.

Lane	Primers	Product	Expected size (bp)
1	P 2 - P 4	Internal	247
2	P 2 - P 3	Internal	134
3	SMART III – P 4	5'	≥ 500
4	P 2 – CDS III	3'	≥ 500
5	P1 – P 4	5' coding sequence	418
6	P 2 – P 5	3' coding sequence	314
7	P 1 – P 5	Full coding sequence	485

Table 4.1. PCR products generated in the amplification of SOD gene sequences from *Nippostrongylus brasiliensis* cDNA. The primer combinations used in each reaction as well as the expected product sizes are indicated.

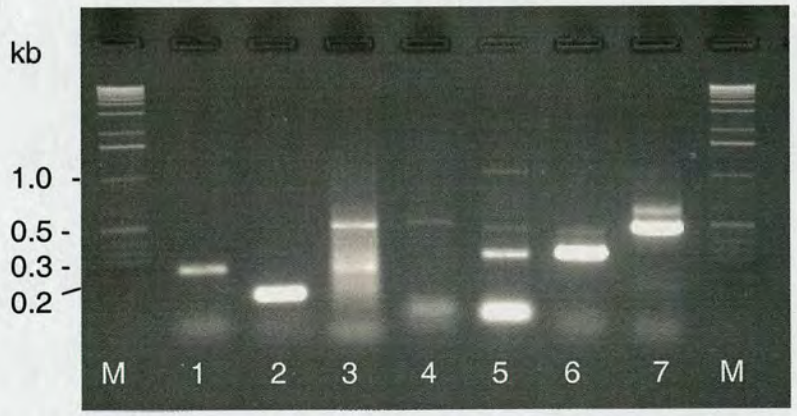


Figure 4.2. PCR products amplified during the isolation of the SOD gene sequence. The lane numbering indicates the product generated using the primer pairs described in Table 4.1 above. PCR amplification generated products of the expected sizes although other less prominent bands were evident in some reactions. Negative controls with no added cDNA gave no products (not shown). M = Molecular weight marker.

4.2.3 SOD gene nucleotide sequence

Sequencing of amplified SOD products was performed as described in Chapter 2.2.9. Multiple reads of products cloned in pGEM-T, covering the span of the gene sequence, were performed to eliminate sequencing errors. These sequence reads were aligned and a full-length contiguous sequence determined. Alignment produced a 650 nucleotide, contiguous sequence, shown in Figure 4.3. The sequence contained a putative open reading frame (ORF), a polyadenylation signal and a polyadenylated tail. A previously determined *N. brasiliensis* SOD sequence (Young, 1996) had an extended 5' sequence with a possible ORF of around 50 bases.

GACGCGTCACAAAGAATGAGCAACCGAGCAGTGGCCGTGTTGAGAGGTGATGCC
GGAGTTACTGGGACGGTGTGGTTCAGTCAGGACAAGGAATCGGACCCGTGTGTG
ATCAAGGGCGAAATCAAGGGTCTGTCCCCTGGTCTTCACGGCTTCCATGTGCAT
CAATACGGTGATTCGACCAACGGCTGTATCTCTGCTGGACCTCACTTTAACCCC
TTCAACAAGACCCATGGAGGTCCGAAGGATGAGGTGCGCCATGTTGGAGACCTC
GGCAACGTGGAAGCCGGAGCCGATGGTGTTGCTCATTTCGAGATCACTGACCAT
ATGGTGAAGATTCATGGTGTGAACACGGTTGTCGGACGTTCGCTGGTGGTTCAT
GCCGGAACTGACGACCTTGGCAAAGGAGTCGGTGAGAAGAAGGAGGAGTCGCTG
AAGACCGGAAACGCCGGTGCTCGCAGCGCCTGTGGAGTGATCGCCATCGCTGCT
CCCCAGTGACTAGGCAGCCTCCTGGCCCACATCTGATTTCCAAACTTATTGTGG
TAATGTAATGCGAGTTGTTGTTCTACCTATTATGTTTTCATTACACATTATCGT
TATATTTGAAGACAGTTATACAAGTACCCTTAATAAATTTAATCAATAAAAAAA

Figure 4.3. The contiguous SOD gene sequence derived from alignment of a number of PCR amplified *Nippostrongylus brasiliensis* SOD sequences. Key: **underlined bases** – putative ORF; – Start codon; – Stop codon; – poly-adenylation signal; – cDNA 3' poly-adenylated tail.

4.2.4 Predicted SOD protein sequence

The ORF encoded a 159 amino acid (aa) protein with a predicted sequence shown in Figure 4.4. Analysis of this protein (using Prosite; http://ca.expasy.org/prosite/) revealed two regions characterised as Cu / Zn SOD signatures. The first of these contains the copper atom, binding site and the second a conserved cysteine involved in disulfide bonding.

Homology analysis, using BLAST search and ClustalW alignment (available http://www.ebi.ac.uk/), revealed a high level of homology between this protein and other nematode SODs as demonstrated in Figure 4.5. Homology is particularly great between this *N. brasiliensis* sequence and the sequence of the cytoplasmic nematode SOD (SODc), with ~95% similarity to the *H. contortus* SODc sequence. As can be seen in an alignment with other nematode sequences (Figure 4.5), the protein also lacks an N-terminal signal leader sequence, carried by the extra-cellular homologues (SODe) in other nematodes. It did not contain any predicted glycosylation sites.

The single 5' elongated SOD sequence, previously determined (Young, 1996), had a putative, extended ORF of around 20 aa (data not shown) which may have corresponded to the N-terminal leader indicating that this ORF encoded an extracellular form of the enzyme. However, analysis did not identify this region as a signal peptide.

```
MSNRAVAVLRGDAGVTGTVWFSQDKESDPCVIKGEIKGLS  - 40
PGLHGFHVHQYGDSTNGCISAGPHFNPFNKTHGGPKDEVR  - 80
HVGDLGNVEAGADGVAHFEITDHMVKIHGVNTVVGRSLVV -120
HAGTDDLGKGVGEKKEESLKTGNAGARSACGVIAIAAPQ  -159
```

Figure 4.4. The predicted *Nippostrongylus brasiliensis* SOD protein sequence translated from the *Nippostrongylus brasiliensis* SOD gene sequence.
Key: ■ - Cu / Zn superoxide dismutase signature 1; ■ - Cu / Zn superoxide dismutase signature 2; **underlined residues** – amino acids conserved with *Haemonchus contortus* sequence and involved in Cu / Zn ion binding and catalyses.

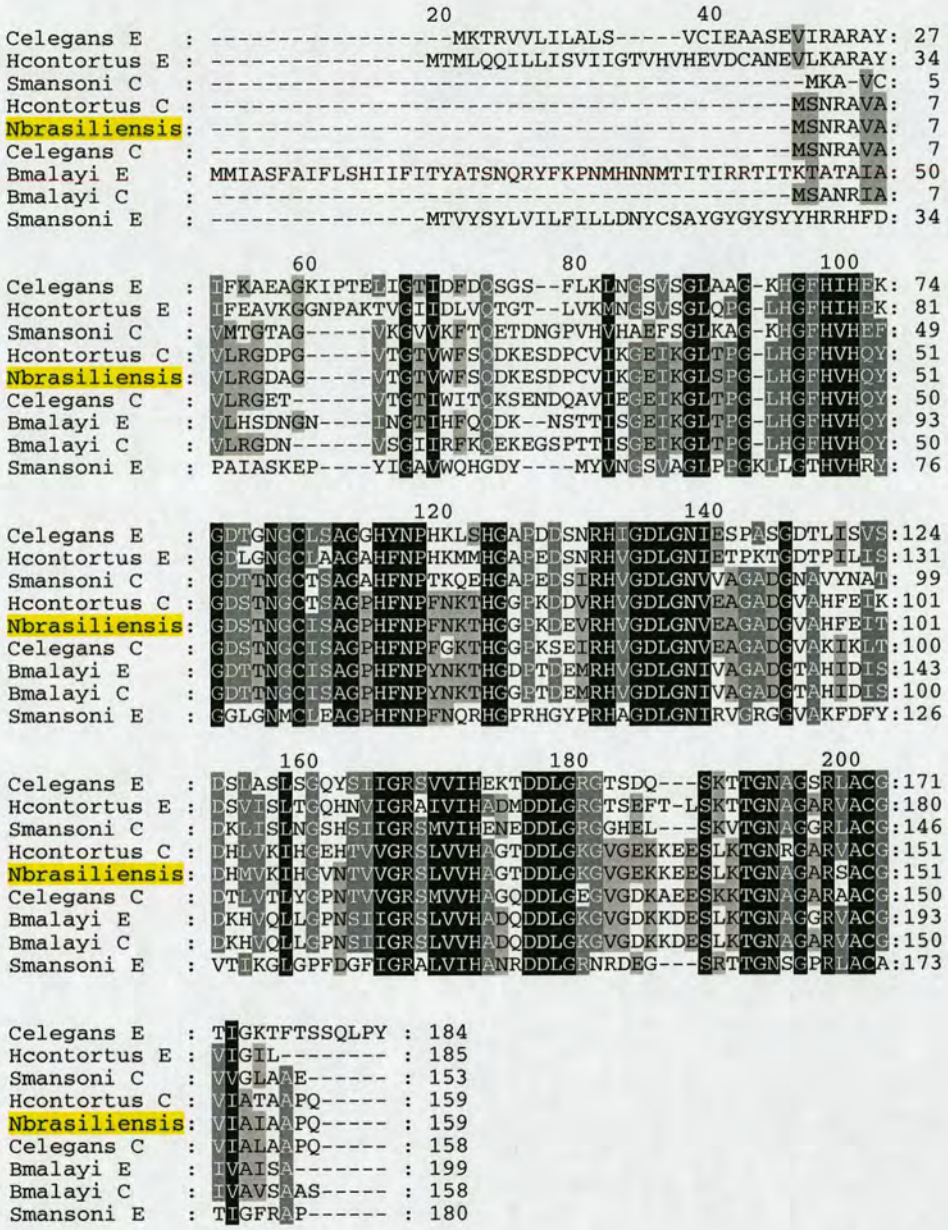


Figure 4.5. The predicted *Nippostrongylus brasiliensis* SOD protein sequence aligned against other known nematode SOD protein sequences from: *Caenorhabditis elegans*; *Brugia pahangi*; *Schistosoma mansoni* and *Haemonchus contortus*. The level of amino acid homology is indicated by shading: ■ – 100%; ▒ – 80%; ▒ – 60 %. Suffix C and E refer to cytoplasmic and extra-cellular homologues of the enzyme respectively.

4.2.5 Phylogenetic analysis

SOD sequences from nematode and other species were used to construct a phylogenetic tree including the putative *N. brasiliensis* sequence. This was carried out using the Phylip program (available online at www.genebee.msu.su). As shown in Figure 4.6, the *N. brasiliensis* SOD groups with other nematodes SODc, and is distinct from SOD of other species and nematode SODe.

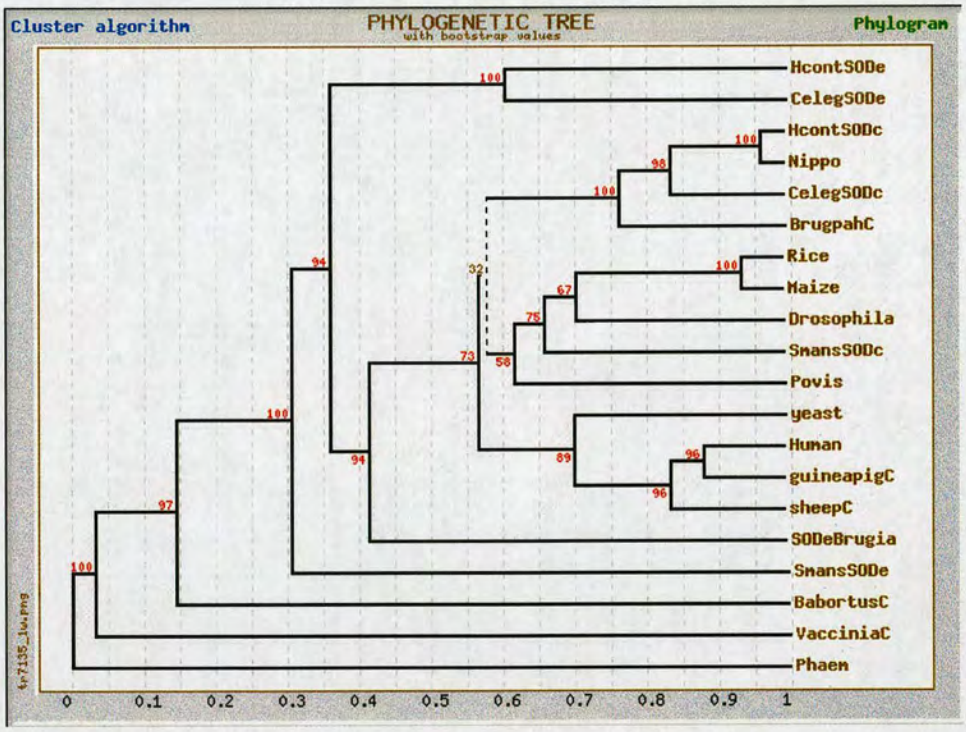


Figure 4.6. A phylogenetic tree constructed from SOD sequences from a range of species. Branch length indicates the divergence between sequences. The tree illustrates the grouping of the *Nippostrongylus brasiliensis* SOD with the SODc of other nematodes and distinctly from the nematodes SODe and the SOD of other phyla.

4.2.6 *In vitro* expression of *Nippostrongylus brasiliensis* SOD

Following sequence analysis the SOD coding sequence was cloned in pET22b+ expression vector as described in Chapter 2.6.3. Constructs were sequenced with vector primers to confirm that inserts had been cloned in frame (data not shown). Protein expression was induced in suitable bacterial cultures and cell fractions were prepared for analysis of expressed proteins. A protein of approximately 21 kDa was abundant in cell fractions from samples of cells containing the SOD insert and absent from vector alone controls as shown in Figure 4.7, panel A, lanes 1 and 2. This size coincided with the predicted size of the *N. brasiliensis* SOD protein based on sequence analysis. Importantly, all of the soluble protein fractions (the culture supernatant consisting of the medium with secreted proteins, periplasmic and soluble cell fractions) as well as the insoluble fraction contained this protein. The recombinant protein was also recognised on a Western blot of bacterial cell fractions by serum raised against *H. contortus* SODc and was not present in the vector alone control (Figure 4.7, Panel B, Lanes 1, 2 & 3 respectively). Gels developed for SOD activity also showed zones of activity consistent with this protein band in samples containing SOD insert and was absent from the pET22b+ alone control (see below).

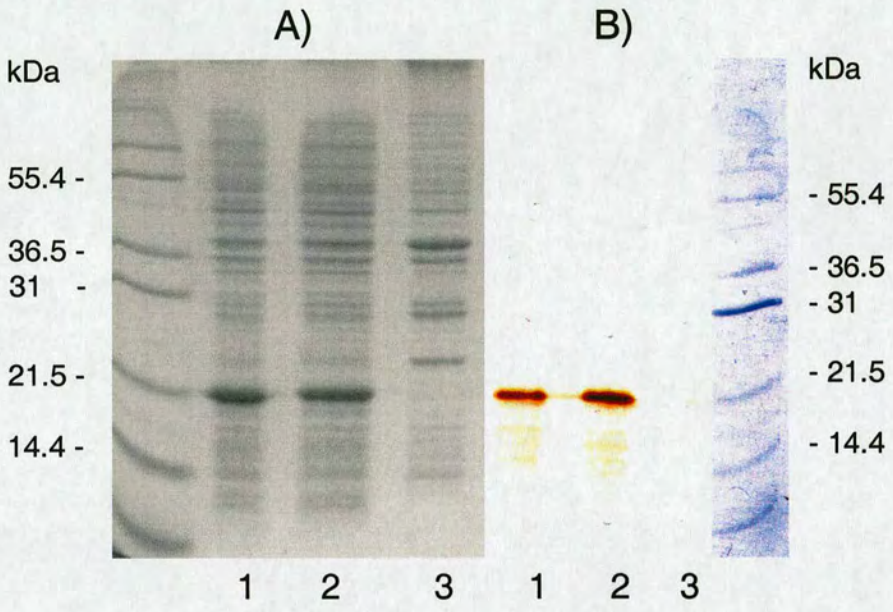


Figure 4.7. Panel A, Coomassie blue stained PAGE gel analysis of: 1) Soluble bacterial cell fraction and 2) insoluble bacterial cell fraction, from cells transformed with the SOD insert, 3) Soluble bacterial cell fraction from cells transformed with pET22b+ only (control). Panel B is an immunoblot of a similar gel to Panel A probed with rabbit anti-*Haemonchus contortus* SOD antiserum. The brown banding indicates the recognition of recombinant SOD from the cell fractions by the serum.

4.2.7 Purification of SOD by ion exchange chromatography

Following detection of the recombinant SOD protein in the soluble fractions of the bacterial culture a purification procedure was devised for the SOD protein to remove the bulk of the bacterial proteins. Recombinant SOD was purified from 100 ml of media fraction by low pressure ion exchange column chromatography, as described in Chapter 2.6.7. The purification was carried out using a Mono Q Sephadex column equilibrated with 10 mM Tris / HCl pH 7.5. Proteins were eluted with a 0 - 30 mM NaCl gradient and the recombinant SOD eluted in a relatively purified and enriched fraction at a concentration of between 5 - 10 mM NaCl as shown in Figure 4.8. Following purification the SOD containing fraction was de-salted using a Sephadex G25 column and eluted in 10 mM Tris / HCl pH 7.5.

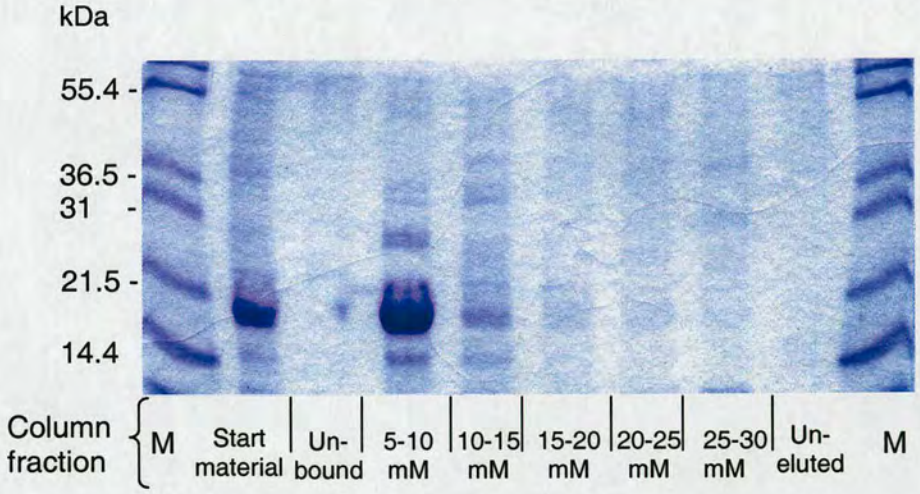


Figure 4.8. Coomassie stained PAGE gel, showing fractions eluted from Mono Q Sephadex column at varying NaCl concentrations during the purification of recombinant SOD. The recombinant SOD is evident as a prominent band at 20kDa in the 5-10 mM NaCl fraction. M = Protein size markers.

4.2.8 SOD activity

The activity of the recombinant enzyme was determined by xanthine SOD assay in the manner of Jones and Suttle (1981) as described in Chapter 2.4.1. The purified recombinant enzyme was active and at a concentration of 200 μg/ml had an activity of 7.6 U/ml. This was approximately three times greater than the media fraction from which the recombinant enzyme had been purified and ten times that of the equivalent cell fraction of the vector control.

SOD activity was also visualised on non-reducing PAGE gels by specific staining in the manner of Beauchamp and Fridovich (1971) as described in Chapter 2.4.3. As can be seen in Figure 4.9, a zone of activity was visible at around 20 kDa, slightly larger than the size predicted for the recombinant enzyme. This zone of activity was inhibited by the addition of 2 mM EDTA, a chelating, metallo-enzyme inhibitor. A zone of activity was present higher up the gel and was only partially inhibited by the addition of EDTA. This may be a Manganese (Mn) - SOD or another free radical scavenging enzyme of the host bacteria.

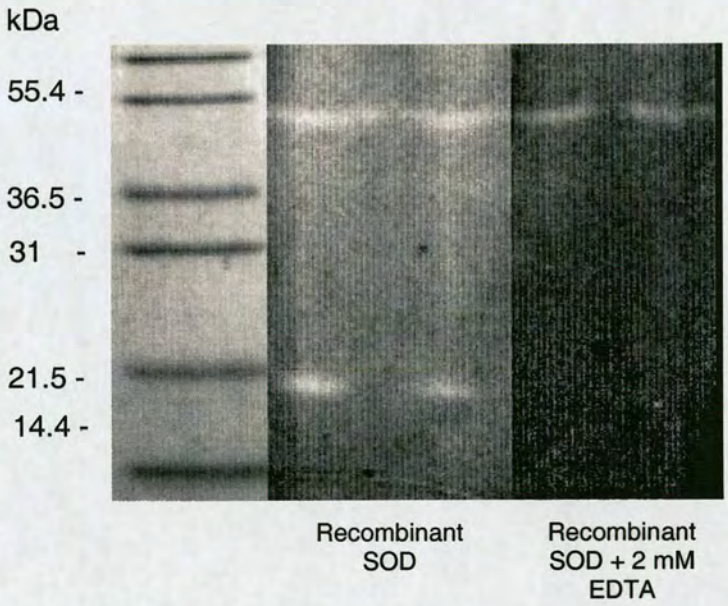


Figure 4.9. A PAGE gel of the *Nippostrongylus brasiliensis* recombinant SOD protein stained for SOD activity. Enzymically active SOD protein is visible as white/clear patches against the mauve background. SOD activity at 20 kDa was inhibited by the addition of 2 mM EDTA a chelating metalo-enzyme inhibitor.

4.2.9 Recognition of recombinant SOD by hyper-immune serum

Recognition of recombinant SOD by serum IgG from hyper-immune rats was tested by Western blot, as described in Chapter 2.3.5. Recombinant SOD and a control of *N. brasiliensis* S1 extract, separated by PAGE, were transferred to PVDF membrane, which was subsequently probed with serum from one rat repeatedly infected with *N. brasiliensis* L3. The blot was then developed for IgG.

An example blot is displayed in Figure 4.10. The IgG from hyper-immune serum recognised the recombinant SOD, present at approximately 21 kDa, and weakly recognised several other proteins in the SOD fraction (lane 2). There was no staining to indicate that the serum recognised an equivalent sized SOD protein in the S1 fraction, although several higher molecular weight bands were recognised (lane 3). These high molecular weight bands corresponded in size to AChE, which are known to be immunogenic in this infection (Jones and Ogilvie, 1972). However, the immune serum did not recognise recombinant AChE on the same gel (lane 1). In a repeat of this experiment, using sera from a different hyper-immune rat, there was no staining indicating recognition of the recombinant SOD or AChE (data not shown).

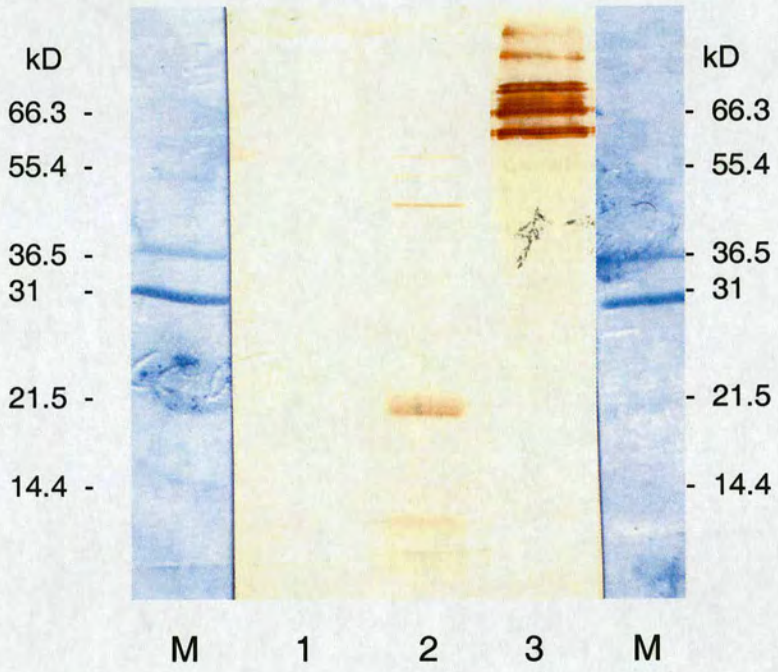


Figure 4.10. Recombinant AChE (lane 1), recombinant SOD (lane 2) and *Nippostrongylus brasiliensis* S1 fraction (lane 3) blotted on PVDF and probed with serum from a hyper-immune rat. The brown banding indictes recognition of the recombinant SOD and high molecular weight proteins in the S1 fraction by IgG in the immune serum. (M= molecular weight markers).

4.2.10 Native SOD enzyme expression

Western blot

Expression of the native enzyme by adult and L3 stages was studied by Western blot. Somatic protein preparations from adult and L3 stages were separated by PAGE and blotted as described previously (Chapter 2.3.5). The Western blots were then probed with serum raised against the *H. contortus* SODc enzyme and previously shown to recognise *N. brasiliensis* SOD (data not shown). These were then developed with ECL+Plus as described in Chapter 2.3.5. As demonstrated in Figure 4.11, panel B, the anti-*H. contortus* SODc serum recognised two protein bands on blots of adult *N. brasiliensis* somatic proteins. These are visible as a doublet with the lower band present in a greater amount than the upper and may represent cytosolic and extra-cellular forms. Two bands were recognised also in the L3 extract. Again these may be cytosolic and extra-cellular forms with the lower, stronger band separating at a similar size to the adult lower band. The upper band appears to separate at a slightly different size to that of the adult indicating that this may be a third different isoform (Figure 4.11). In the corresponding Coomassie stained PAGE gel (panel A), the dark band between 21 and 14 kDa (lane 2, adult extract) is probably globin, previously described as the most abundant protein in *N. brasiliensis* adult somatic extracts (Blaxter *et al.*, 1994).

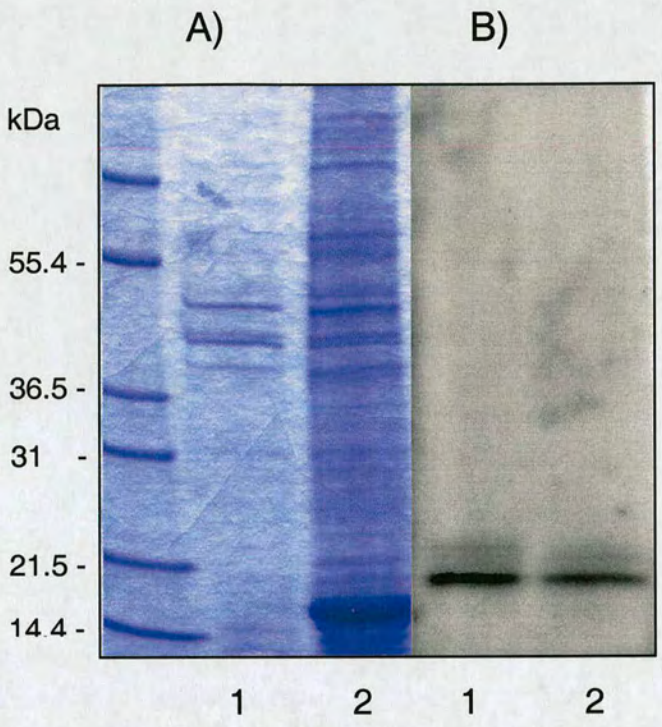


Figure. 4.11. Panel A) a Coomassie stained PAGE gel and B) the corresponding Western blot of; 1) L3 and 2) adult *Nippostrongylus brasiliensis* somatic extracts developed with anti-*Haemonchus contortus* SODc serum and ECL+ Plus. The dark bands on the blot indicate the presence of SOD isoforms in the somatic extracts.

Detection of SOD mRNA by reverse transcription polymerase chain reaction

Expression of *N. brasiliensis* SODc coding mRNA was also studied in adult and L3 stages by ReverseTranscription (RT) PCR. First strand cDNAs were prepared from total RNA extracted from adult and L3 as described in Chapter 2.2.1. SOD-specific olignucleotide primers were then used to amplify specific sequences from an equal amount (100 ng) of each cDNA. Products were then visualised, or detected, by separation on agarose / EtBr gels. As can be seen in Figure 4.12, specific SODc products were amplified from both adult and L3 stages as well as the positive control (SMART cDNA from adult worms), but not from the negative control, a PCR reaction that contained no template DNA. This suggests that, as shown in the previous experiment, this SOD was transcribed in both adult and L3 stages. Accurate quantification was not possible due to the lack of a constitutively expressed control. However, the relative brightness of the product from the L3 was greater than that from the adult, for the same amount of template. This might suggest that the level of expression was elevated in the L3. A truly quantitative RT PCR would be required to confirm this result.

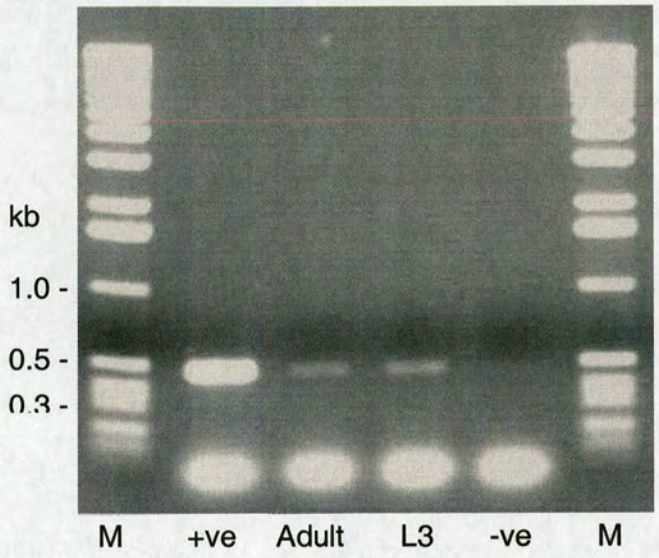


Figure 4.12. An agarose / EtBr gel showing SOD specific PCR products amplified from adult and L3 cDNA. The same amount of template was added to each reaction, therefore the similar brightness of the products indicates the amplification of similar amounts of SOD from the cDNA from both L3 and adult worms. +ve) positive control, –ve) negative control. M = molecular weight marker.

4.3 Discussion

SOD specific sequences were amplified by RACE PCR from adult *N. brasiliensis* cDNA, using primers directed towards the conserved active site region (Figure 4.2). These sequences were confirmed as encoding a SOD by performing homology searches of DNA and protein databases, an analysis which revealed a high level of homology between the putative *N. brasiliensis* SOD sequence and SOD sequences of other nematodes (Figures 4.4 and 4.5). The ORF encoded a 159 aa protein shown in Figure 4.4. Sequence analysis of this protein revealed 2 regions characterised as Cu / Zn superoxide dismutase signatures and 9 aa conserved with the *H. contortus* sequence and involved with metal ion binding and catalysis (Liddell and Knox, 1998). The protein was thus confirmed as a *N. brasiliensis* Cu/Zn SOD, a prediction confirmed by the activity detected when the protein was expressed in bacteria.

BLAST searching and Clustal-W alignment also showed a high level of homology between this protein and other nematode SODs (Figure 4.5). The high homology of the DNA and amino acid sequences to cytoplasmic isoforms (~95% similarity to the *H. contortus* SODc cDNA sequence) strongly suggested that this *N. brasiliensis* sequence encodes a cytoplasmic form of the enzyme. This was supported by further analysis of the amino acid sequence, which showed that it did not contain an N-terminal leader sequence usually indicative of protein secretion. These leader sequences, of around 20 aa, are common to extra-cellular SOD of other nematodes and enable the extra-cellular transport of the enzymes (Liddell and Knox, 1998; Henkle-Duhrsen *et al.*, 1997). A phylogenetic analysis (Figure 4.6) also grouped the *N. brasiliensis* enzyme with other nematodes SODc and distinct from extra-cellular SODs.

One partial single sequence from previously determined SOD sequences had a putative extended ORF at the 5' end encoding 20 additional aa, the entire sequence of which could correspond to the extracellular form of the enzyme (Young, 1996). That an extracellular form may be present was indicated by Western blot analysis of L3 and adult parasite extracts (Figure 4.11). Release of SOD by *N. brasiliensis* during *in vitro* culture has also been described (Batra *et al.*, 1993).

In addition, analysis of this partial sequence showed some amino acid changes from the derived consensus indicating that it may be a separate gene product. The elongated N-terminal sequence was not, however, recognised as a signal leader peptide and protein modelling (not shown) suggested that the tertiary structure of the two putative proteins would not differ significantly due to the sequence differences. It was therefore decided that although this extended sequence might be the extra-cellular form of the *N. brasiliensis* enzyme, the cloned, full length SODc would be used for the vaccination trials.

The sequenced enzyme was expressed as a recombinant protein by cloning in pET22b+ expression vector (Figure 4.7), in a manner previously used to express SOD from *H. contortus* (Liddell and Knox, 1998). Soluble, recombinant SOD was identified by Western blot and purified from the bacterial culture media by ion exchange chromatography. A purified / enriched fraction eluted from the Mono Q Sephadex column at around 7 mM NaCl. The recombinant protein was detected in this fraction by PAGE gel at 21 kDa with Coomassie staining and confirmed as SOD by Western blot (Figure 4.8).

The enzyme activity of the purified recombinant SOD fraction was measured by a xanthine/xanthine oxidase superoxide generator assay and the activity was 7.6 U/ml, approximately three times that of the source fraction and ten times that of the control (pET22b+ alone). Enzyme activity in this assay is determined by measuring the inhibition of colour formation which results from the reaction of superoxide, generated by xanthine/xanthine oxidase, with a tetrazolium dye (Jones and Suttle, 1981). Activity was also demonstrated at around 21 kDa on PAGE gels stained for activity of the enzyme. The activity was inhibited by EDTA indicating that it is dependent on metal ions in the manner of other Cu / Zn SOD (Liddell and Knox, 1998; Henkle-Duhrsen *et al.*, 1994).

The recombinant enzyme was recognised by sera from a hyper-immune rat (Figure 4.10). This suggests that the native protein is immunogenic and also suggests that an antibody response to this protein may be involved in protection, as this animal was, following several infections, solidly immune. The recognition of SOD may however be incidental, given the range of proteins these animals are exposed to during infection. As the recombinant was not recognised by sera from a different

hyper-immune rat, it may not be important in the protective immune response. This finding also suggests that differences in the immune response occur between individual infected rats.

Expression of the native SOD enzyme was investigated in adult and L3 stages of *N. brasiliensis*. Western blots prepared from adult and L3 somatic extracts were probed with serum raised against *H. contortus* SODc (Liddell and Knox, 1998) and previously shown to recognise the *N. brasiliensis* enzyme (Figure 4.11). These blots showed two isoforms were present in both adult and L3 stages, confirming a previous study, which indicated the presence of several SOD isoforms in different life stages (Knox and Jones, 1992). The lower protein band was the same size in both stages and is present in a greater amount in both as judged by the depth of staining. The size of the upper band from each stage appeared to differ in size, with the L3 form being slightly larger than the adult. By analogy to other SODs, the upper band in L3s and adults is likely to be an extracellular form of the enzyme as these contain additional N-terminal leader sequence (Liddell and Knox, 1998; Henkle-Duhrsen *et al.*, 1997). However, cDNA sequence analyses failed to conclusively identify a sequence encoding this form of the enzyme. The difference in size of this band in L3 and adults may indicate the presence of distinct isoforms of the enzyme with differential expression between life stages, similar to those demonstrated in an earlier study (Knox and Jones, 1992). This suggestion would require further analysis, one approach being the determination of the N-terminal sequences of the two protein bands.

The expression of mRNA coding for the SODc was also investigated by RT PCR. Specific *N. brasiliensis* SODc products were amplified from both adult and L3 stages, supporting the previous finding from protein analysis that this form is expressed in both stages (Figure 4.12). The PCR was not quantitative due to the lack of a constitutively expressed control for normalisation of PCR products on agarose gels. However, the band produced from the L3 cDNA appeared brighter on an agarose gel than that from the adult cDNA for the same amount of template material. This suggests that there was an increased copy number of SOD cDNA in the L3 and therefore an increased level of expression of the enzyme in the L3. Increased levels

of SOD activity were previously demonstrated from L3 larval stages of several species of nematode parasite (Knox and Jones, 1992).

As the mRNA detected in this study is considered to code the cytoplasmic form of the protein and the L3 is more active and therefore presumably more metabolically active, the findings could be interpreted as increased SOD expression in the L3 to counteract metabolic stress. A fully quantitative PCR with a constitutively expressed control against which to normalise the amount of PCR product would be required to test this result.

In conclusion, this chapter has described the successful isolation and expression of a cDNA encoding a putative cytosolic SOD from adult *N. brasiliensis* that is also present in L3 larvae. The SOD was expressed as an enzymically active recombinant protein in bacteria and the following chapters describe its evaluation as a vaccine candidate against *N. brasiliensis* in the rat.

Chapter 5 – The development of assays to monitor the immune responses of the rat

5.1 Introduction

Previous studies have characterised the immune responses of the host rat during infection with the parasitic nematode *N. brasiliensis* as described in Chapters 1 and 3 (reviewed; Rothwell, 1989). The response in general can be characterised as a Th-2 immune response based on the pattern of cytokine and cellular responses seen. Th-2 hypersensitivity responses are a common feature of helminth infections and are characterised by antigen-specific IgG, IgE and IgA with eosinophils and mast cells infiltrating the site of infection (Rothwell, 1989). Cytokines, especially IL–4 and –5, are often implicated in the development of protective immune responses (Urban *et al.*, 1992; Finkelman *et al.*, 1997).

Cellular responses during GI nematode infection include the accumulation and activation of mast cells, basophils, eosinophils, goblet cells and lymphocytes (Miller, 1984). While some or all of these cell types might have a function in the expulsion of the infection, accumulation of others may be incidental. Any of the cell types commonly accumulating during a challenge infection may, however, be used as a marker of infection indicating the stimulation by the parasite of a cellular response in the intestinal tissue.

Prior to the vaccination trials described later (Chapter 6, 7 and 8), assays for the measurement of immune markers of infection were devised and optimised. These tests were to allow the level of antibody, cellular and cytokine response in vaccinated animals to be compared to controls and to provide data for comparison of immune responsiveness between trials.

Changes in antibody levels during infection are well documented with increases in IgG, IgA and IgE being associated with expulsion in this system (Jarrett and Haig, 1976; Jarrett and Bazin, 1977). These associations are discussed in Chapter 1 and 3 and it is clear that the stimulation of antibody responses might be an objective of vaccination. An appropriate method for the measurement of host

antibody responses in sera and tissue is enzyme linked immunosorbent assay (ELISA; Wedrychowicz *et al.*, 1983). Secondary antibodies for a panel of rat antibody isotypes were obtained and ELISAs developed and optimised for the measurement of parasite, or immunising antigen-specific antibody levels during this study.

As noted above, mast cells accumulate in large numbers in the intestinal mucosa of the rodent host during infection with *N. brasiliensis* (Miller, 1984). When activated, they release their granule contents including a protease (RMCP II; Miller *et al.*, 1983b). Mast cell protease levels rise both systemically and in mucosal tissue during both rodent and sheep nematode infections, this rise correlating with worm expulsion (Miller *et al.*, 1983b; Huntley *et al.*, 1993; Douch *et al.*, 1996). RMCP II measurement is therefore a useful marker of mast cell activation during nematode infection. Mast cell proteases have been ascribed a variety of functions, one of which is to act on epithelial cell tight junctions to increase the epithelial permeability to enable the passage of macromolecules such as immunoglobulin into the intestinal lumen (McDermott *et al.*, 2003).

RMCP II levels can be measured in serum and tissues by ELISA and a commercially produced kit is available for this purpose. This kit was tested for the measurement of RMCP II levels in the sera and small intestine tissue of infected animals.

The expression of cytokine mRNA in rats during infection with *N. brasiliensis* has been studied previously by RT PCR (Matsuda *et al.*, 1995; 1999). These experiments confirmed the increased expression of Th-2 cytokines during infection, in particular expression of IL -3, -4 and -5 increases correlating with expulsion, while Th-1 cytokine expression is depressed. The utility of RT PCR to investigate cytokine expression levels during vaccination trials was assessed in a series of experiments.

5.2 Results

5.2.1 Optimisation of serum antibody ELISA

The serum antibody ELISA was optimised using hyper-immune and control rat sera, as these were likely to show large differences in specific antibody level. Initially, several dilutions of coating antigen and a dilution series of primary serum from 1 in 20 to 1 in 20480 were tested. These dilutions of primary serum have previously been used in measurement of sheep antibody levels by a similar ELISA method. Secondary antibodies specific for; total IgG, IgG1, IgG2a, IgA and IgE, were then tested at a range of dilutions (based on the manufacturers recommendation) to determine the most appropriate dilution.

Coating antigen concentration was not found to effect the signal level in the ELISA, at all the dilutions tested (data not shown). Accordingly, it was subsequently used at the highest dilution tested, 1 μg/ml. The dilution series of primary serum chosen also gave an appropriate reduction in signal level through the dilution range required to allow accurate quantification of antibody level (data not shown).

The OD from a series of ELISAs with hyper-immune and control sera, using a dilution series of secondary antibody are shown in Figure 5.1, panel A – E. As shown in all of the graphs, large differences in antibody level existed between sera from the negative control and hyper-immune rats. This is shown by the higher OD of the immune samples at equivalent dilutions to control samples.

The results indicated that the secondary antibodies were effective through all of the dilution range used for each, as shown by the tight matching of the curves from either control or immune sera. One exception was the IgG secondary antibody, which showed some decrease in the signal seen with an increase in dilution (Panel A).

Antibody titre during this study was determined by comparison of the antibody level of a test sample, against the level in the negative control sample. This was the dilution of the test sample that had the equivalent antibody level of a 1 in 20 dilution of the negative control. In the results summarised in Figure 5.1, this is

equivalent to the point on the graphs where the immune OD crosses the control 1 in 20 dilution OD level.

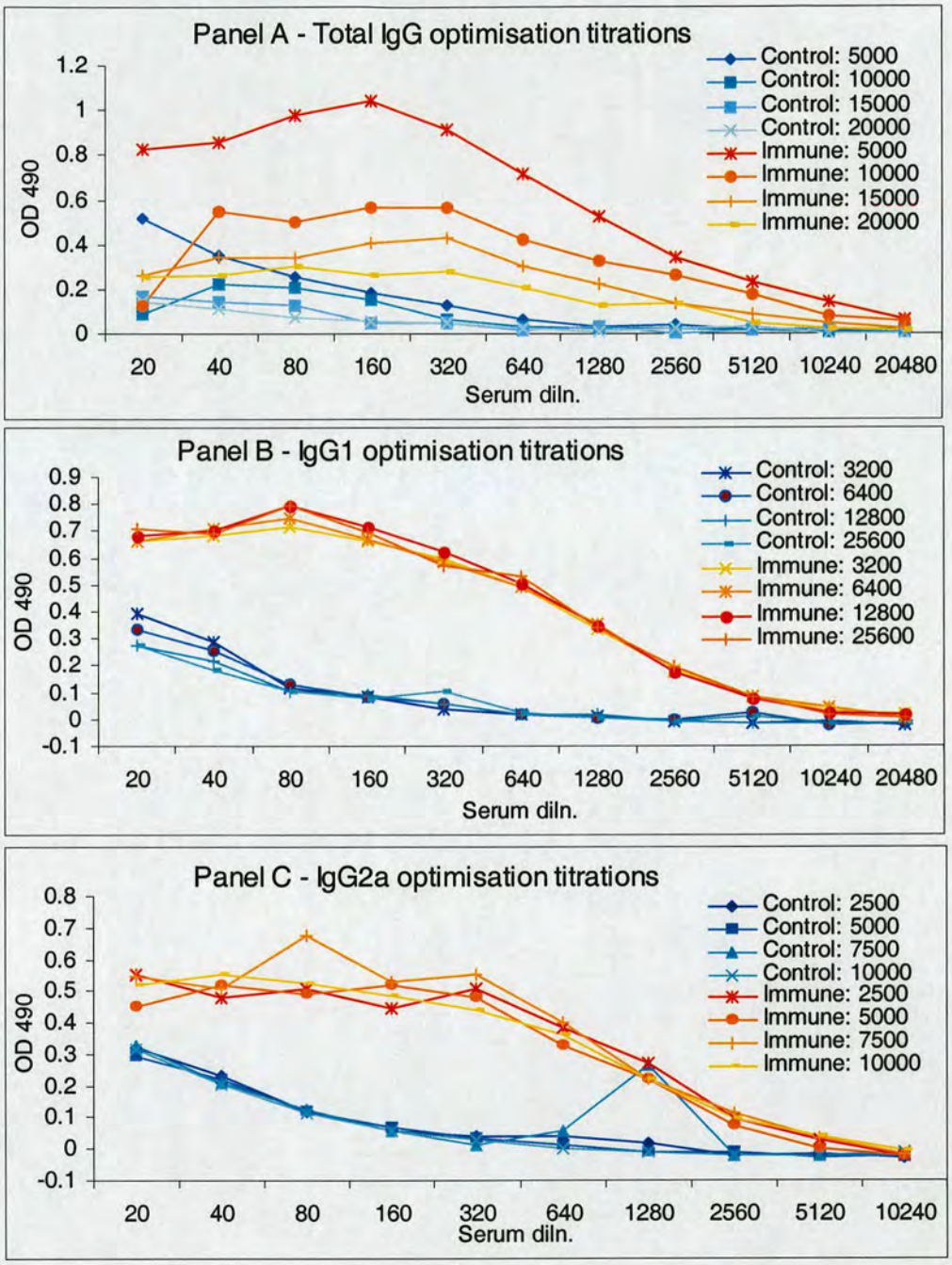


Figure 5.1 Panel A-C. The effect of secondary antibody dilution on ELISA (IgG, IgG1 and IgG2a) optical density. The curves represent the OD of ELISA performed using dilution series of pooled immune or control sera and differing dilutions of secondary antibody. The dilution of secondary antibody did not appear to affect the performance of the ELISA in the range tested for IgG1 and IgG2a detection antibodies. For total IgG the OD reduced with secondary antibody dilution and this antibody was accordingly subsequently used at the highest dilution tested.

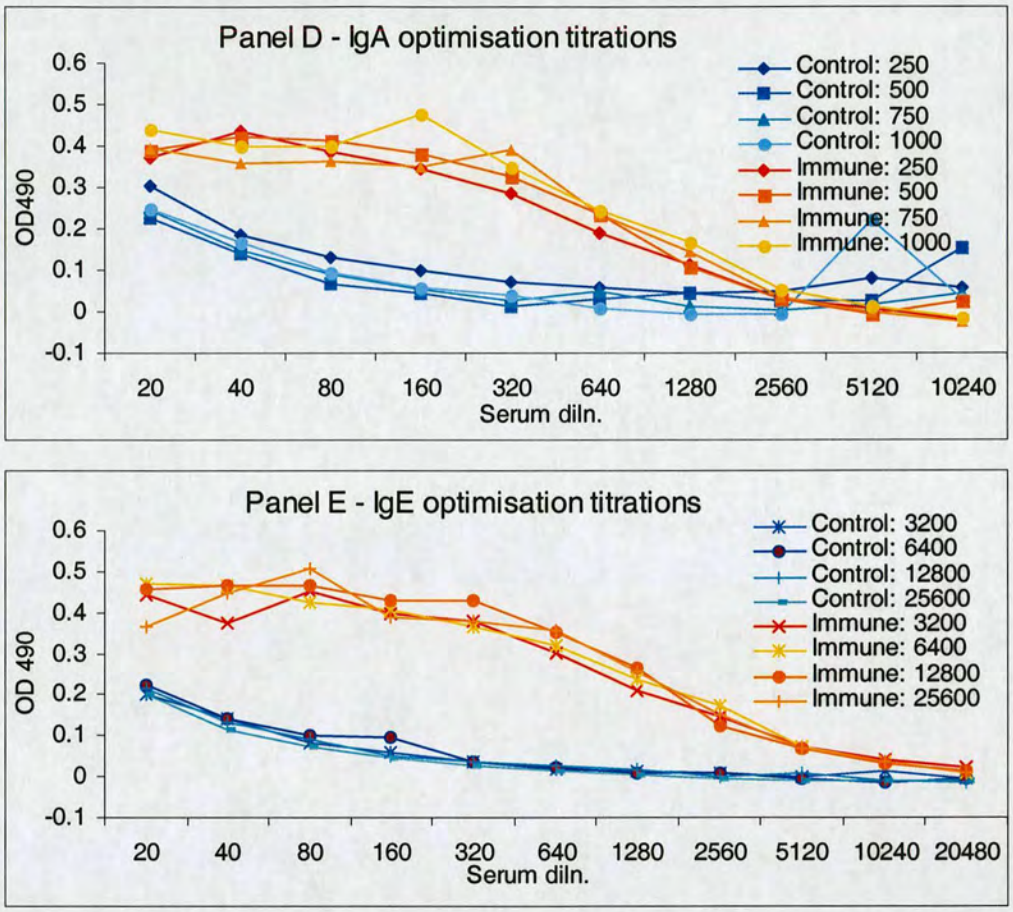


Figure 5.1 Panel D and E. The effect of secondary antibody dilution on ELISA (IgA and IgE) optical density. The curves represent the OD of ELISA performed using dilution series of pooled immune or control sera and differing dilutions of secondary antibody. The dilution of secondary antibody did not appear to affect the performance of the ELISA in the range tested for IgA and IgE detection antibodies.

5.2.2 Optimisation of mucosal antibody ELISA

The ELISA for the detection of antibody in mucosal homogenates was initially tested under the same range of primary antibody dilutions as the sera, using the optimised secondary antibody levels. Mucosal homogenates were prepared from animals at 7, 14 and 23 DPI with 2,000 L3 and control animals, as described in Chapter 2.7.

The ELISA results for the mucosal homogenates are shown in Figure 5.2. Differences in mucosal antibody level between infected and control animals were detected by this ELISA method for IgG, IgG1, IgG2a and IgE. Slight differences were also apparent in the IgA results although these were somewhat masked by the high background OD at higher dilutions. These data indicated that levels of antibody were much lower in the mucosa than in sera and that a lower range of primary antibody dilutions would be more suitable. A doubling dilution series of the mucosal homogenates in a range from neat (10 mg/ml) to 1 in 1,024 was therefore used in subsequent assays.

Titres could be calculated for these results in the same manner as for the sera results, by comparison to 1 in 20 of the neat (10 μg/ml) control (data not shown).

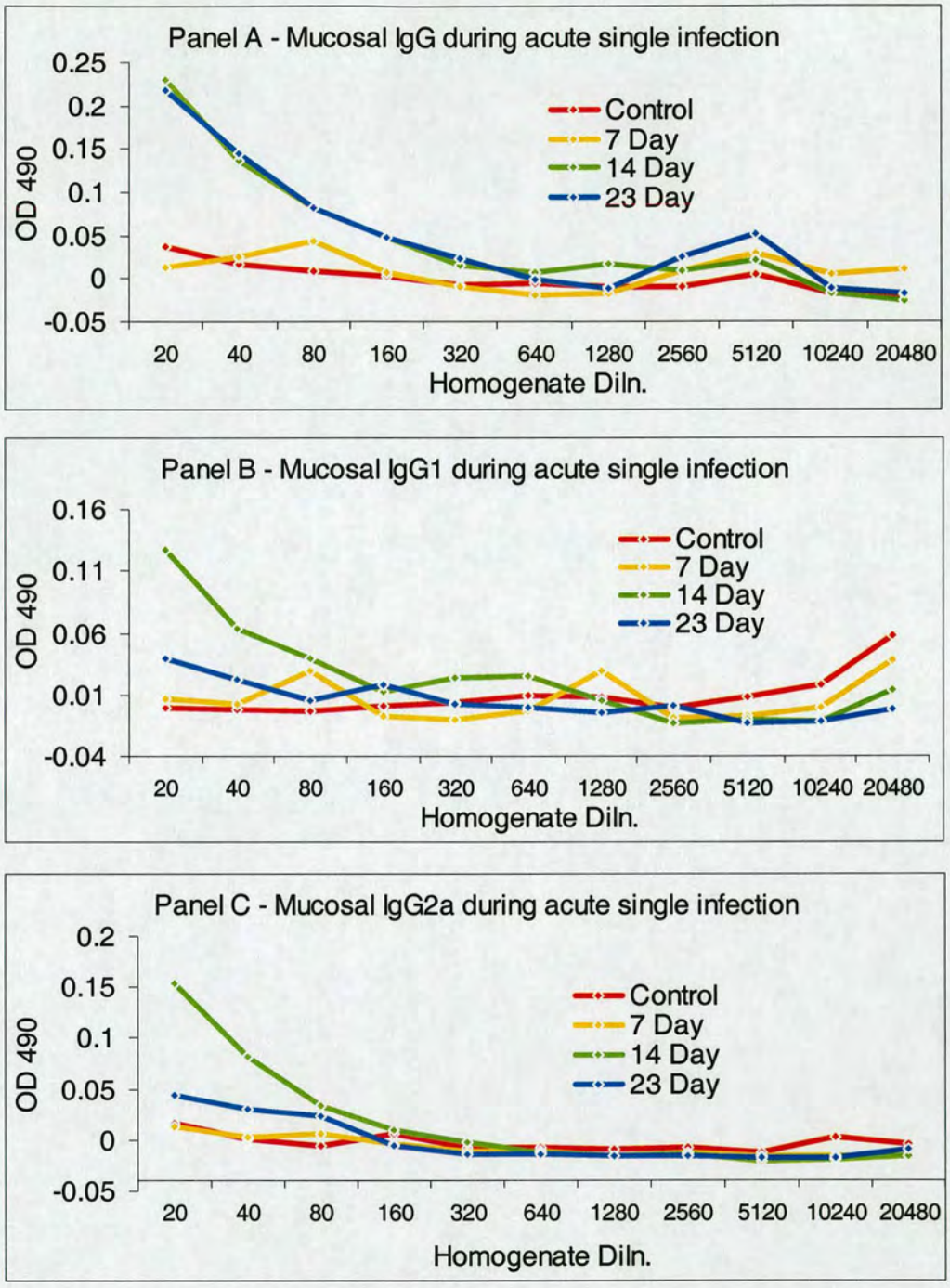
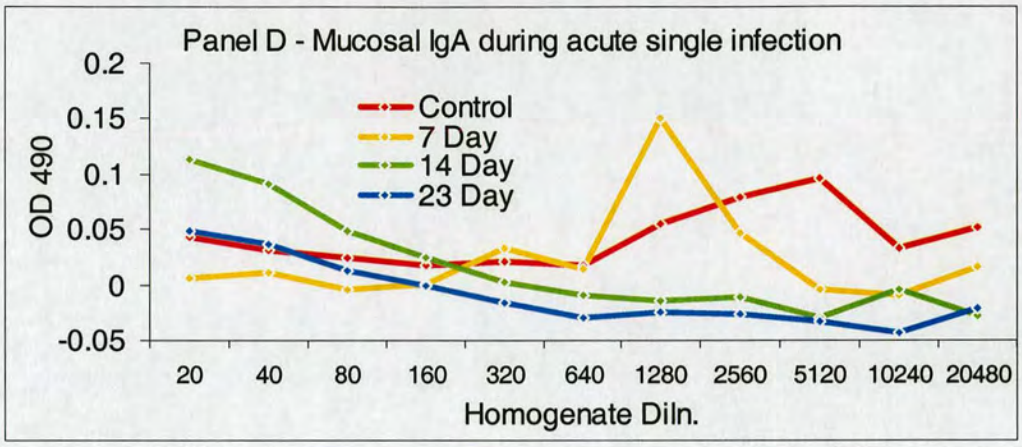


Figure 5.2. The effect of primary antibody (mucosal homogenate) dilution on ELISA (IgG, IgG1and IgG2a) optical density, with samples from four timepoints during infection. The data represent the OD of ELISA performed on a dilution of mucosal homogenate from an individual animal at each timepoint. Differences in the antibody level were observed between the different timepoints.



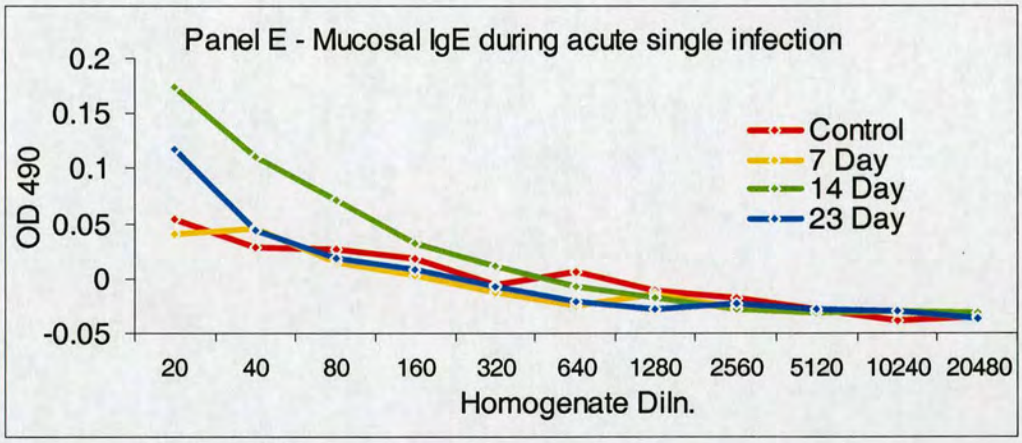


Figure 5.2 (continued). The effect of mucosal homogenate dilution on ELISA (IgA and IgE) optical density, with samples from four timepoints during infection. The data represent the OD of ELISA performed on a dilution of mucosal homogenate from an individual animal at each timepoint. Differences in the antibody level were observed between the different timepoints.

5.2.3 Measurement of rat cytokine expression by RT PCR

RNA extraction

Total RNA was extracted from rat spleen, intestinal mucosa and mesentery collected at regular time-points during infection with 2,000 L3 as described in Chapter 2.7.2. These samples were chosen for their relevance at the site of infection (mucosa), draining lymph nodes (mesentery), or their role in systemic immune response (spleen). Prior to cDNA synthesis, samples of RNA were separated on agarose / EtBr gels for analysis. One such gel is shown in Figure 5.3, panel A. A smear of mRNA was evident to above 3 kb with no evidence of degradation. This sample is typical of the quality of RNA seen during the tissue extraction from spleen, mucosa and mesentery.

cDNA synthesis

Samples of RNA (as shown in Figure 5.3, Panel A) from different tissues and at different time-points were used in the synthesis of cDNA in the first step of the RT PCR, in the manner described in Chapter 2.7.2. Following first strand synthesis, a sample of cDNA was analysed on agarose / EtBr to check the quality and yield. One sample is shown in Figure 5.3, panel B. The cDNA was visible as a smear to over 3 kb with products between 0.5 and 1.5 kb predominating.

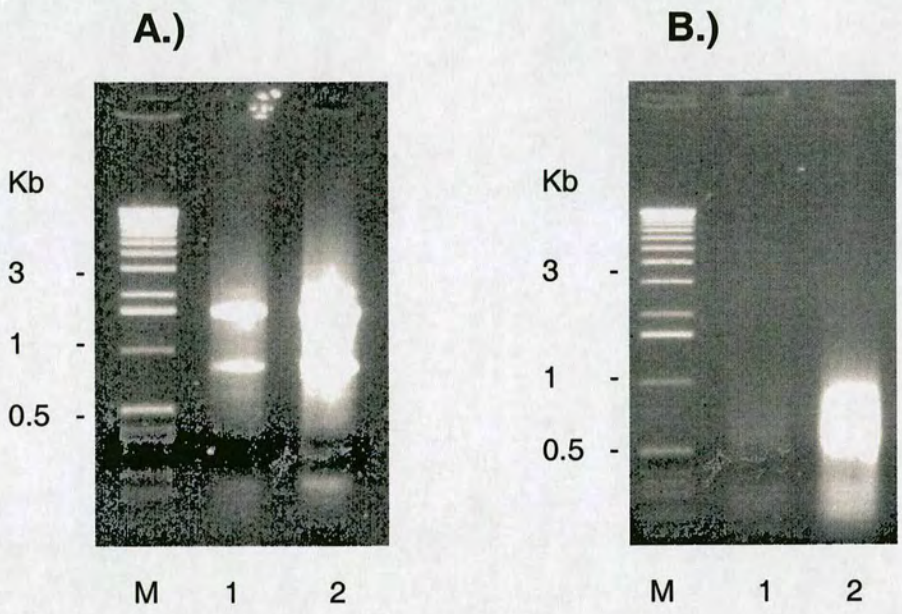


Figure 5.3. A typical agarose / EtBr gel of A.) RNA and B.) the corresponding cDNA from rat mucosa at 1.) 14 days post infection and 2.) 0 days (Control). The smear extending through the size range and the lack of low molecular weight degradation products indicates the integrity of the RNA. (M = molecular weight marker).

The detection of cytokine expression in rat tissues using RT PCR

Amplification of cytokine specific sequences by PCR was performed using positive control or test rat tissue cDNA with the primers shown Chapter 2.7.2. Typical results, obtained with rat spleen mRNA, are shown in Figure 5.4. Only the amplification with β-actin primers, a constititively expressed mRNA species, produced a product, confirming the viability of the cDNA template.

Also shown (Figure 5.5) are the products of PCR with primers for IL-4 and IL-5 and a dilution series of a positive control cDNA template. These reactions produced products of the expected size with visible bands produced even at low levels of template cDNA. The positive result from the IL-4 and IL-5 primers with a positive control template suggests that the PCR reaction works well with the addition of sufficient template.

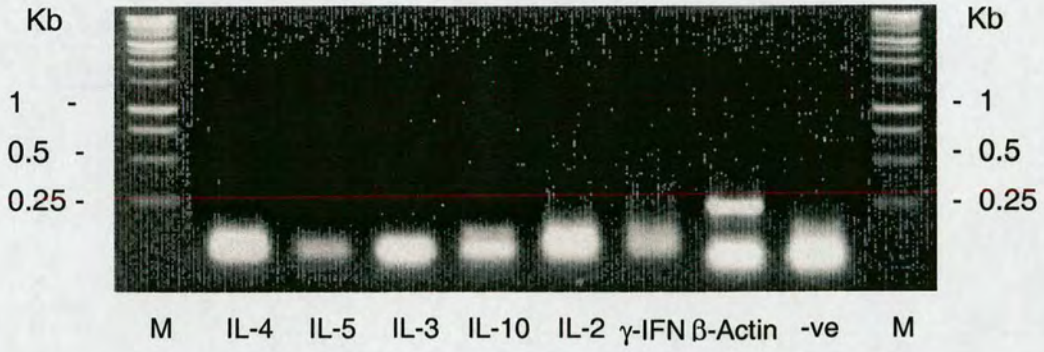


Figure 5.4. A typical agarose / EtBr gel of PCR products amplified using a panel of rat cytokine primers and *N. brasiliensis* infected rat spleen cDNA template. Only primers specific for β-Actin (a constituitively expressed control), consistently amplified a product from test samples. M= kilobase marker.

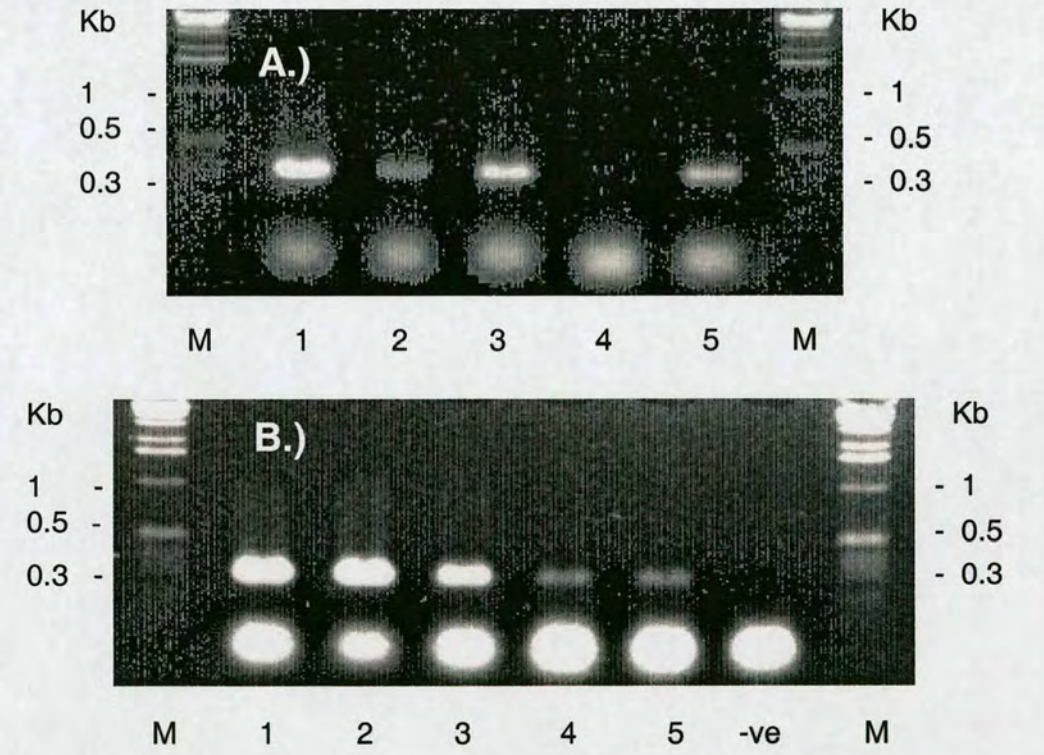


Figure 5.5. Agarose / EtBr gels of PCR products specific for A.) IL-4 and B.) IL-5 produced using a positive control template at a range of dilutions. Lanes 1-5 contain the products from reactions performed using doubling dilutions from 1 in 2 to 1 in 32 of a 10x positive control from the Maxim Biotech MPCR kit. Products were amplified to a high dilution of template DNA.

The detection of cytokine expression in the intestinal mucosa using a Multiplex PCR

The detection of rat cytokine expression in tissue samples was also attempted using a commercial multiplex PCR kit as described in Chapter 2.7.2. This kit contained positive control template for optimising the PCR with GAPDH as an internal control in the primer mix. Some results are shown in Figure 5.6. Products of the expected size were produced for all cytokine primer sets in the panel with the addition of the positive control template. The internal control, GAPDH, gave a product of the expected size in all of the samples tested confirming the quality of the cDNA. In the infected tissue samples, bands were evident that were approximately the same size as those of the cytokines in the positive control and some variation was seen in the appearance and intensity of these bands between samples at different time-points. However, replicate PCR often failed to reproduce these results and when some of these bands were cloned and they were not found to match the expected cytokine sequences (data not shown).

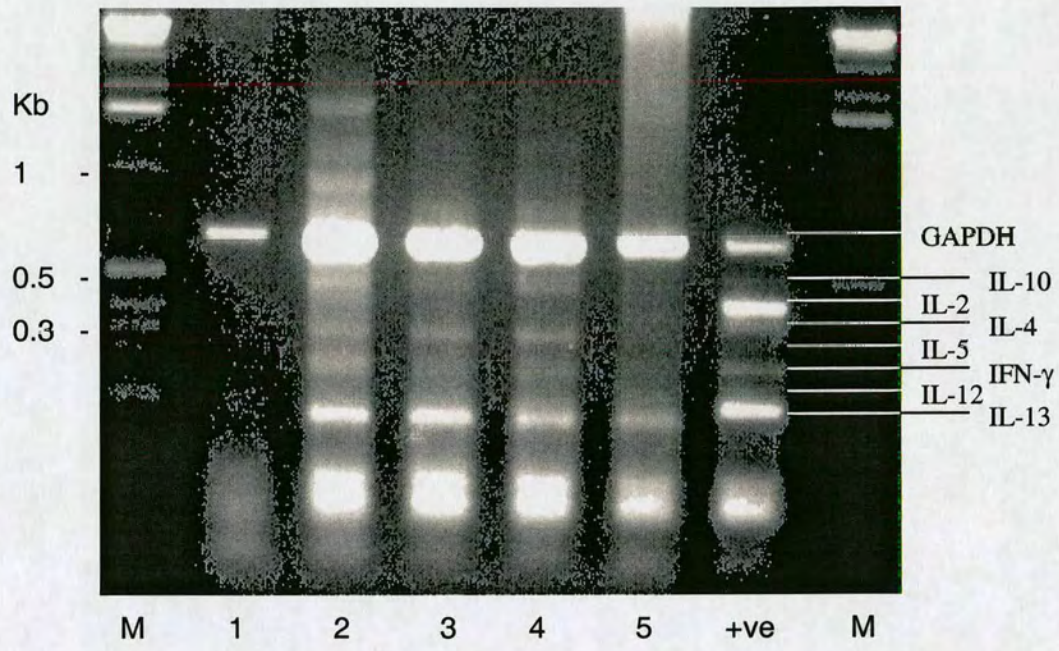


Figure 5.6. Agarose EtBr gel showing PCR products produced in a multiplex PCR with cDNA template from rat small intestinal mucosa at; 1.) 0 DPI; 2.) 7 DPI; 3.) 9 DPI; 4.) 11 DPI and 5.) 14 DPI. Some products appeared to correspond in size to cytokine specific products, labelled above in a positive control PCR (+ve). However, more accurate sizing indicated that these products were not the same. (M = kilobase marker).

5.3 Discussion

This chapter describes the development and optimisation of assays for the measurement of immune markers of infection and immunisation. Assays for the measurement of specific antibody and RMCP II by ELISA and cytokine production by RT PCR were developed. These were used to measure specific antibody and cellular responses in the vaccination trials described in later chapters.

The data from the optimisation of the antibody ELISA indicated that, as expected, large differences existed in the antibody level present in the sera of control and hyper-immune rats. These large differences allowed the optimisation of the ELISA with regard to coating antigen, primary and secondary antibody dilution. Standard operating concentrations of coating antigen and the primary antibody dilution were thereby set.

The OD from ELISA with a series of dilutions (Figure 5.1) showed that, with the exception of the total IgG secondary antibody, the secondary antibodies were effective through all of the dilution ranges used for each. Accordingly, the secondary antibodies were used in subsequent ELISAs at the highest dilution in the tested range. The total IgG secondary antibody was used at the lowest dilution tested as this gave the strongest signal.

The levels of antibody present in hyper-immune serum samples could also be calculated as a titre whereby the level was expressed as the dilution necessary to reduce the OD to the level of a 1 in 20 dilution of the control sera. This was to be the standard method for expressing all subsequent antibody ELISA data. These titres (data not shown) indicated that the profile of antibody production in hyper-immune animals was similar to that described previously (Jarrett and Haig, 1976; Jarrett and Bazin, 1977), as discussed in Chapter 3. The highest response was in total IgG with this dominated by the IgG1 component. IgG1 was the antibody isotype associated with protection from infection in this system (Jones *et al.*, 1970). High IgE titres were also stimulated but the IgA response was less.

Differences were also evident in the levels of the different antibody isotypes present in the small intestine mucosa during the course of an acute single infection (Figure 5.2). These differences were not so large as those seen between immune and

naïve sera, but were measurable. The use of samples taken at various time-points through a challenge with 2,000 *N. brasiliensis* L3 allowed the optimisation of the ELISA with regard to the dilution of primary antibody, in this instance the soluble tissue homogenate.

As well as allowing optimisation of the mucosal ELISA, this test gave mucosal antibody titres from several time-points through a primary infection as described previously in Chapter 3. These changes in mucosal antibody matched the changes seen in the sera through the course of the infection, in a manner similar to those previously observed in this system (Poulain *et al.*, 1976a; Sinski and Holmes, 1977; Wedrychowicz *et al.*, 1983). The highest responses were in IgG and IgE with a reduced but still visible response from IgA. Both IgE and IgA isotypes peaked earlier (14 DPI) in infection compared to IgG which remained elevated during the period of sampling.

Despite repeated attempts, no products were produced from test rat cDNAs when PCR were performed using cytokine primers. Positive control PCR with the IL-4 and IL-5 primer pairs gave good signals from low levels of template indicating that the deficiency was not in the PCR (Figure 5.5). The visualisation of RNA and cDNA prior to detection of cytokine species also indicated good quality template production from all samples and at all time-points, as judged by their separation on agarose / EtBr (Figure 5.3). Further evidence of the quality of the template was provided by the positive control PCR, which amplified β-actin products from all of the tested samples (Figure 5.4). These data suggest that cytokine transcripts were not present in sufficient quantity to be detected by this method.

A commercial multiplex PCR kit was also tested for its ability to detect cytokine mRNA. Once again the internal control, in this case GAPDH, was amplified from samples at all time-points indicating the viability of the template cDNA and the appropriateness of the PCR conditions (Figure 5.6). Several other bands were also amplified of several sizes, some of which were similar to the sizes of specific cytokine bands produced in the positive control reaction. Differences were also apparent between samples of the same tissues collected at different time-points.

Accurate sizing of these bands however showed that many of them were not of sizes matching those expected for the cytokine bands, suggesting non-specific

amplification at the high template and low stringency conditions used. Several of the bands were cloned and sequenced and the results indicated that these sequences were not the expected cytokines.

It must therefore be concluded that RT PCR failed to accurately, or repeatedly, detect cytokine mRNA from the test samples as described previously (Matsuda *et al.*, 1995). This may have been due to the absence of cytokine mRNA or extremely low levels of such mRNA in the tissues tested. Some variation in the amount of cytokine mRNA expressed does exist between rat strains and it may be that the strain used in these experiments expresses only low levels of these species (Uchikawa *et al.*, 1996). The RNA extraction was also performed on whole tissues rather than on dissected lymph nodes or purified cells, which may be more appropriate sources of material for the detection of cytokine expression by RT PCR (Matsuda *et al.*, 1995; Matsuda *et al.*, 1999). As the assay was intended for use as a marker of Th-2 responses in the vaccine trials described below, it was decided to use the RMCP II ELISA as an indicator of this response.

To overcome these problems, dissection of specific lymph node tissue, or isolation and purification of lymph node cells might be required. These cell populations will contain most lymphocytes, a major cytokine producing cell population. *In vitro* stimulation of these cell populations with worm antigens might also be required for the production of sufficient transcripts for detection (Matsuda *et al.*, 1999). Improved detection systems such as fluorescent labelled probes could also be employed for low amounts of PCR product as well as more accurate quantification by densitometry.

In summary, the work described in this chapter set out to establish routine and discriminatory methods to monitor the immune response in the vaccination trials described below. ELISAs were established to measure the major antibody isotypes in sera and in intestinal mucosa samples taken at the site of infection. Despite considerable effort, a reproducible PCR-based assay for cytokine expression could not be established. Possible reasons for this and future directions to overcome the problem were addressed briefly in this discussion.

Chapter 6 - The effect of subcutaneous vaccination with recombinant *Nippostrongylus brasiliensis* acetylcholinesterase and superoxide dismutase on the outcome of infection in the rat

6.1 Introduction

As stated in Chapter 1, previous attempts at vaccination against several species of nematode have been made using worm AChE. In a Guinea pig / *T. colubriformis* model, no significant level of protection was achieved (Rothwell and Merritt, 1975). However, in this experiment the antigen was given as a single dose sub-cutaneously without an adjuvant, prior to parasite challenge. Guinea pigs were partially protected against *D. viviparus* challenge following subcutaneous immunisation with AChE partially purified from adult ES, the antigen being delivered subcutaneously with Freund's adjuvants (McKeand *et al.*, 1995a; McKeand *et al.*, 1995b). No protection was observed in a subsequent trial in calves but, on this occasion, the AChE was a recombinant of a single isoform of the enzyme (Matthews *et al.*, 2001). In a mixed nematode infection in sheep of *T. colubriformis, H. contortus* and *C. oncophora,* vaccination with *T. colubriformis* AChE led to a 31% reduction in the total worm burden (Griffiths and Pritchard, 1994).

These studies indicate that AChE may be a useful target antigen for vaccination. However, none have systematically examined the effect of route of AChE delivery on the outcome of a subsequent challenge infection. This is one of the aims of the work described in this and following Chapters.

As described in Chapter 1, SOD may play a critical role in parasite survival in the intestinal tract. To date, the protective efficacy of this protein has only been tested using a combined recombinant vaccine comprising the cytosolic and extracellular forms of the protein from *H. contortus* (Liddell and Knox, 1998). A 20% reduction in final worm burdens was noted when vaccinated animals were compared to controls. The vaccinated animals had an elevated faecal egg output, an observation that may indicate that the parasites were exposed to increased

immunological stress. Again, in this trial, antigens were given SC with Quil A as adjuvant.

Most previous experimental vaccinations against GI nematodes have been performed via a systemic route of vaccination (Knox, 2000). The immune response to systemic vaccination is characterised by increases in serum immunoglobulin levels and specifically in IgG (Harlow and Lane, 1988). However, it is likely that the immune responses generated in this way are not the most appropriate in protection against GI nematode infection, which may be modulated by local humoral and cellular responses (Emery *et al.*, 1993). In a previous vaccination experiment using *N. brasiliensis* protection was achieved using somatic antigens immunised sub-cutaneously, but a higher degree of protection was afforded by IP vaccination, a regime that stimulates mucosal as well as systemic responses (Murray *et al.*, 1979). Therefore, the choice of vaccination route can affect the outcome of vaccination against mucosally associated parasites such as *T.colubriformis* or *N. brasiliensis*.

Antibody responses may be characterised as Type (Th)-1 or -2 by the IgG isotype produced. In rats, Th-2 responses are associated with IgG2a and IgG1 and Th-2 responses (Binder et al, 1995) with the IgG2b and IgG2c isotypes (Gracie and Bradley, 1996). The choice of adjuvant and inoculation site may affect the Th-1 / -2 balance of the immune response. As protection against nematode infections is considered to be due to Th-2 polarised effects, this too might affect whether an immune response was protective.

In most previous trials of helminth vaccines an adjuvant has been administered in combination with the immunising antigen to boost the immune response. Freund's adjuvant has been used extensively because it stimulates both humoral and cellular immune responses, but is now considered unsuitable due to its potentially harmful side effects and could not be used for commercial vaccine production purposes (Cox and Coulter, 1997). An acceptable adjuvant is Quil A, which has been used with success in developing anti-helminth vaccines (Knox and Smith 2001). Quil A is a saponin derived adjuvant that boosts immune responses in a non-specific manner but which is considered to primarily induce a high antibody titre (Estrada *et al.*, 2000).

The first vaccination experiment in rats using recombinant AChE and SOD, described in this chapter, was designed to follow a similar regime of systemic vaccination to that commonly used in sheep. In rodents, systemic vaccination may be performed by SC immunisation. This trial was considered to be a test of the immunogenicity of the two candidates as well as determining any protective effect conferred by an immune response. Subsequent trials consequently investigated the effect of immunisation by other routes (Chapters 7 and 8). In this trial a group of animals was immunised with the bacterially expressed recombinant SOD (Chapter 4) in combination with Quil A and another with a *Pichia*-expressed recombinant AChE in combination with Quil A. Both recombinant proteins were enzymically active. Control groups consisted of a group immunised with Quil A adjuvant alone and a non-immunised group. These groups were designated SOD, AChE, Adjuvant and Control groups respectively.

6.2 Results

6.2.1 Observations

In general, the vaccination regime was well tolerated with no obvious side effects. In the group immunised with SOD and Quil A however, serious effects did occur in two animals. In both cases this consisted of bleeding from the nose, with one animal more affected than the other. This implied a respiratory system haemorrhage. The more heavily affected animal was extremely distressed and was euthanised, the other subsequently recovered with no further effects.

6.2.2 Pre-challenge IgG level

The level of antigen-specific antibody in the serum of SOD and AChE vaccinated animals was measured by ELISA 7 days prior to challenge infection, by the method described in Chapter 2.7. The titres of SOD- and AChE-specific antibody compared with control rat serum are displayed in Figure 6.1. The titre of SOD- and AChE-specific antibody was measured also in the Quil A immunised (Adjuvant) animals as a measure of non-specific antibody binding or background titre conferred by the Quil A component of the immunisation alone.

As shown in Figure 6.1, panel A, the SOD immunised animals did not show a SOD-specific IgG response significantly greater than that of the Adjuvant control. The AChE immunised animals showed a statistically significant increase in the level of AChE-specific IgG over the Adjuvant control ($P=0.007$, Figure 6.1, panel B).

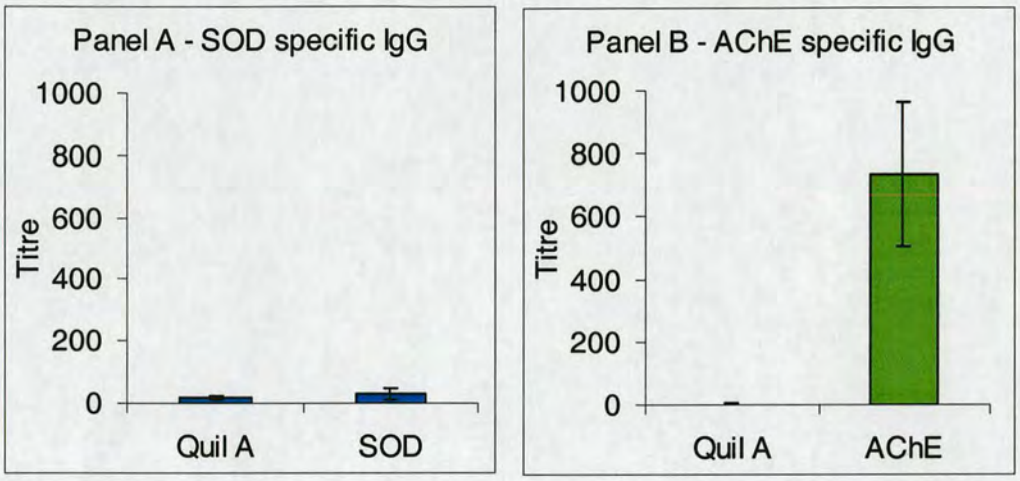


Figure 6.1 Panel A.) the SOD specific and B.) the AChE specific IgG levels in serum from Quil A (Adjuvant), SOD and AChE immunised rats 7 days prior to challenge. The data represent the mean total IgG titre of the 7 animals in each vaccination group measured by ELISA (+/- the standard error).

6.2.3 Faecal egg counts

FEC from control and vaccinated groups are displayed in Figure 6.2, panel A - C. The FEC from the control group was similar to that demonstrated previously for a challenge infection of 25 L3 (Chapter 4). Animals vaccinated with the adjuvant alone showed a similar egg output to the controls (Figure 6.2, panel A).

The results from the group of animals vaccinated with SOD are shown in Figure 6.2, panel B. The FEC from these animals was slightly elevated compared with those of the controls but this was not statistically significant, when tested by analysis of variance (one way ANOVA).

The FEC from the AChE vaccinated group was markedly reduced through the course of the infection. The largest difference occurred around days 6 - 8 post infection, around the peak of infection. This difference in egg output corresponded to a decrease of 48% in the total egg output through the course of infection. The peak in egg output in this group was also later, occurring 8 DPI rather than 7 DPI for the other groups. The reduction in egg counts in this group, however, did not prove to be statistically significant, when tested by one way ANOVA.

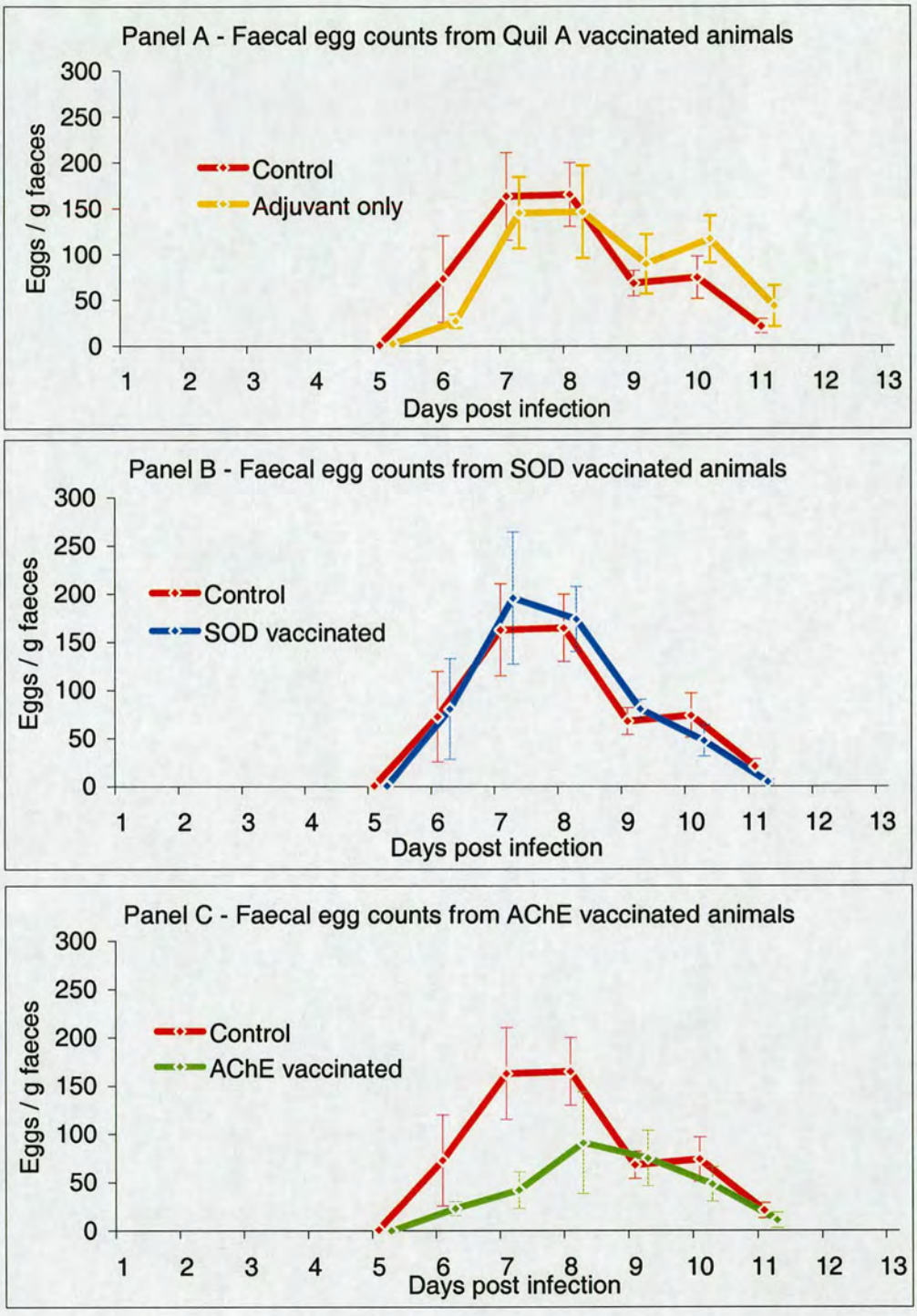


Figure 6.2. The faecal egg count from Control (unvaccinated) and Adjuvant, SOD and AChE vaccinated animals over 11 days following challenge infection with 25 *Nippostrongylus brasiliensis* L3 larvae. The data represent the mean of faecal egg counts performed on three pooled samples from each group at each timepoint (+/- the standard error). Vaccination with AChE reduced the egg output compared with the controls.

6.2.4 Adult worm counts

At the time of sampling, 11 DPI, no adults were detected in the small intestine of any of the animals.

6.2.5 Serum antibody levels

Antigen (SOD or AChE)-specific antibody levels were measured in the serum of the vaccinated animals and controls at the time of necropsy, as described in Chapter 2.7. Results, expressed as titres over the uninfected control, are shown in Figures 6.3, panels A and B.

The levels of specific antibody isotypes in the serum of animals in the Adjuvant alone immunised group was not significantly different from the Control group for either antigen (Figure 6.3, panels A and B). The results from ELISA measuring SOD-specific antibody indicated that low titres of each of the antibody isotypes (IgG, IgG1, IgG2a, IgA and IgE) were present in the sera from the SOD, Adjuvant and Control groups (Figure 6.3, panel A). The SOD vaccinated group did not show significantly higher levels of specific antibody than those seen in the Control and Adjuvant groups.

The antigen specific antibody levels from the serum of AChE vaccinated animals are displayed in (Figure 6.3, panel B). This group had a significantly elevated AChE specific IgG titres compared to the Control and Adjuvant groups ($P=0.002$). Significantly increased serum titres of both AChE-specific IgG1 and IgG2a were detected compared with these controls ($P<0.02$). A significantly increased mean level of IgA and IgE was also observed in serum from this group compared with the control groups ($P<0.02$).

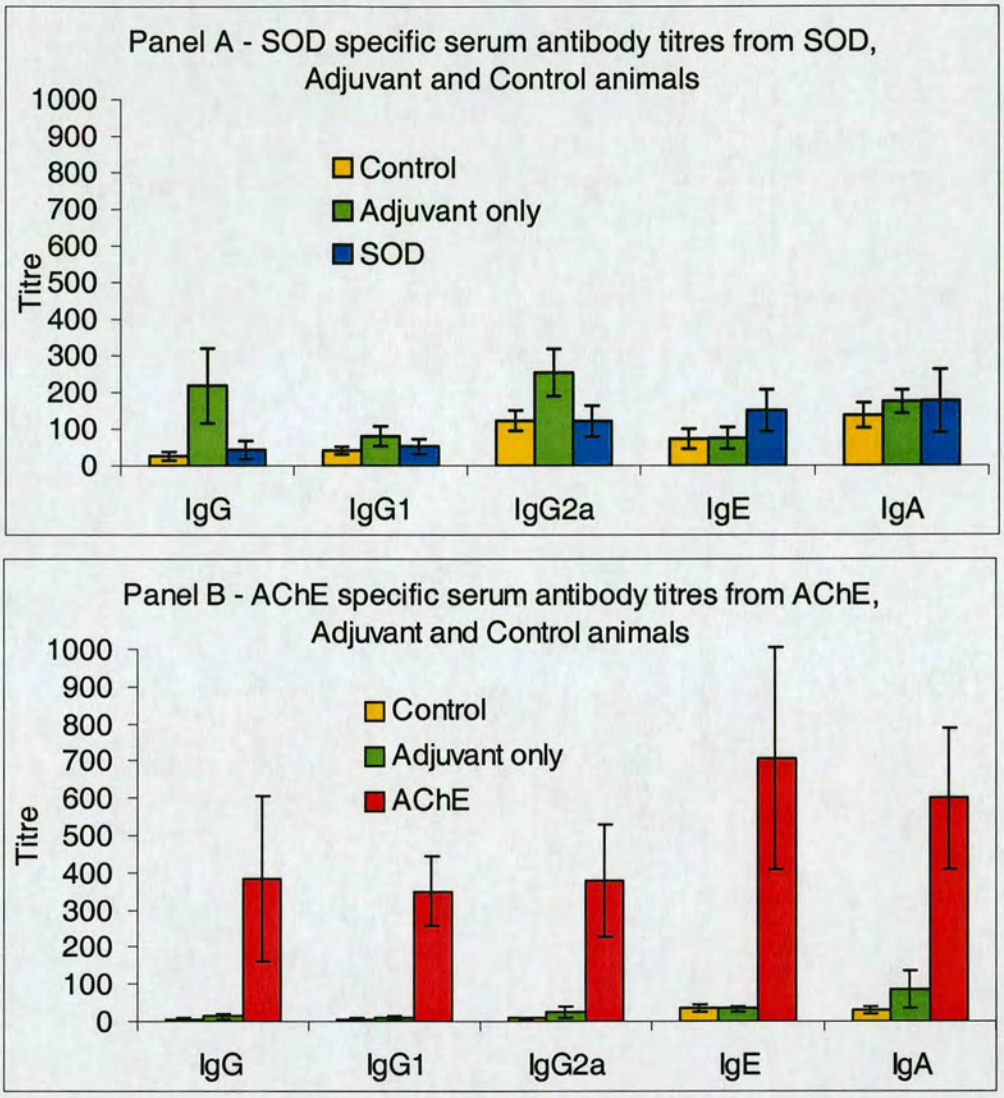


Figure 6.3. Panel A.) The SOD and B.) AChE specific serum antibody response of SOD and AChE vaccinated animals, Adjuvant alone vaccinated and Control (unvaccinated) animals at 11 days following challenge with 25 *Nippostrongylus brasiliensis* L3. The data represent the mean antibody titre of the 7 animals in each vaccination group measured by ELISA (+/- the standard error). A marked difference in the magnitude of the serum antibody response was observed between the groups.

6.2.6 Confirmation of antibody specificity by Western Blot analysis

The specificity of antibody from immunised animals for the immunising antigens was confirmed by Western blot. Blots were performed as described in Chapter 2.3.5 with the antigens, SOD and AChE being transferred to PVDF membrane and probed with primary serum from SOD, AChE or Adjuvant animals. These were then developed for total IgG.

Blots are displayed in Figure 6.4, and as demonstrated in lanes 1 and 2, SOD on the membrane was not recognised by IgG in sera from SOD vaccinated or Adjuvant control animals. SOD was recognised weakly by serum from 2 individual animals on subsequent blots performed in the same manner (data not shown). AChE was recognised by sera from AChE vaccinated animals, reflecting the increased IgG titre (Figure 6.4, lane 3). AChE was not recognised by sera from Quil A (Adjuvant) immunised animals (Figure 6.4, lane 4).

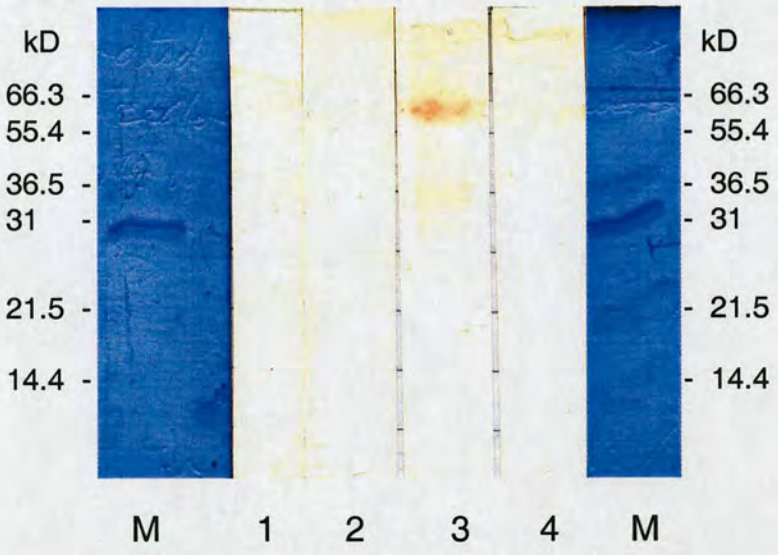


Figure 6.4. A Western blot of recombinant SOD (lanes 1 and 2) and recombinant AChE (lanes 3 and 4) probed with serum IgG from; SOD vaccinated (lane 1), AChE vaccinated (lane 3) or Adjuvant vaccinated animals (lanes 2 and 4). Recombinant AChE was recognised by serum from AChE vaccinated but not from Adjuvant vaccinated rats. SOD was not recognised on the blot. (M denotes molecular weight markers).

6.2.6 Mucosal antibody

Using a mucosal antibody ELISA, as described in Chapter 2.7.1, no specific antibody was detected in the mucosa of vaccinated and control animals at necropsy 11 DPI.

6.2.7 Serum and mucosal mast cell protease levels

Mast cell protease (RMCPII) levels were measured in the serum and mucosa of vaccinated animals and the results are displayed in Figure 6.5. RMCP II levels in serum from Control, Adjuvant alone and SOD vaccinated animals was higher than those previously seen in uninfected controls (Chapter 3), ranging from 800-1,000 ng/ml. The level of RMCP II in serum from the AChE vaccinated group was significantly lower than these groups at around 400 ng/ml (P=0.001).

Levels of RMCP II in the mucosa were around 50 – 100 ng/mg soluble protein in the Control and SOD vaccinated groups. The level in the AChE group was significantly elevated above these at around 400 ng/mg soluble protein (P=0.002). The levels in the Adjuvant alone group were also elevated above the Control and SOD at around 700 ng/mg soluble protein, however the variation in this group was also high as indicated by the large standard error and the difference was not significant.

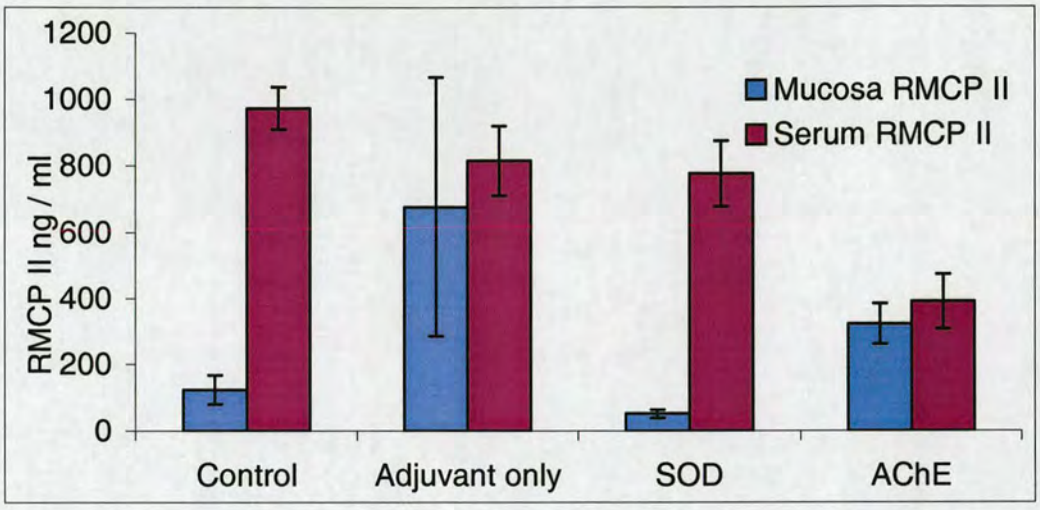


Figure 6.5. The levels of mucosal and serum RMCP II from Control (unvaccinated) and Adjuvant SOD and AChE vaccinated animals at 11 days following challenge infection with 25 *Nippostrongylus brasiliensis* L3. The data represent the mean RMCP II level of the 7 animals in each vaccination group measured by ELISA (+/- the standard error). Some differences in the levels of RMCP II in each compartment were observed between the groups.

6.2.8 The effect of antibody on acetylcholinesterase activity

The effect of serum from vaccinated animals on the activity of recombinant AChE was investigated by incubating AChE with sera from individual animals, then separating the proteins on a native PAGE gel and staining for esterase activity.

Several zones of activity were visible when recombinant AChE was incubated with serum from control animals, Figure 6.6, panel A. The prominent upper band could be attributed to endogenous esterase activity in the serum (serum lane) with the lower band being due to the recombinant AChE (AChE lane). When the recombinant AChE was incubated with sera from animals vaccinated with recombinant AChE, the pattern changed (Figure 6.6, panel B). In this case the activity in the recombinant AChE band, for several individuals (lanes 10 and 12), was decreased and in all animals, a zone of activity high on the gel appeared indicating a slowly, or non-migrating band of activity.

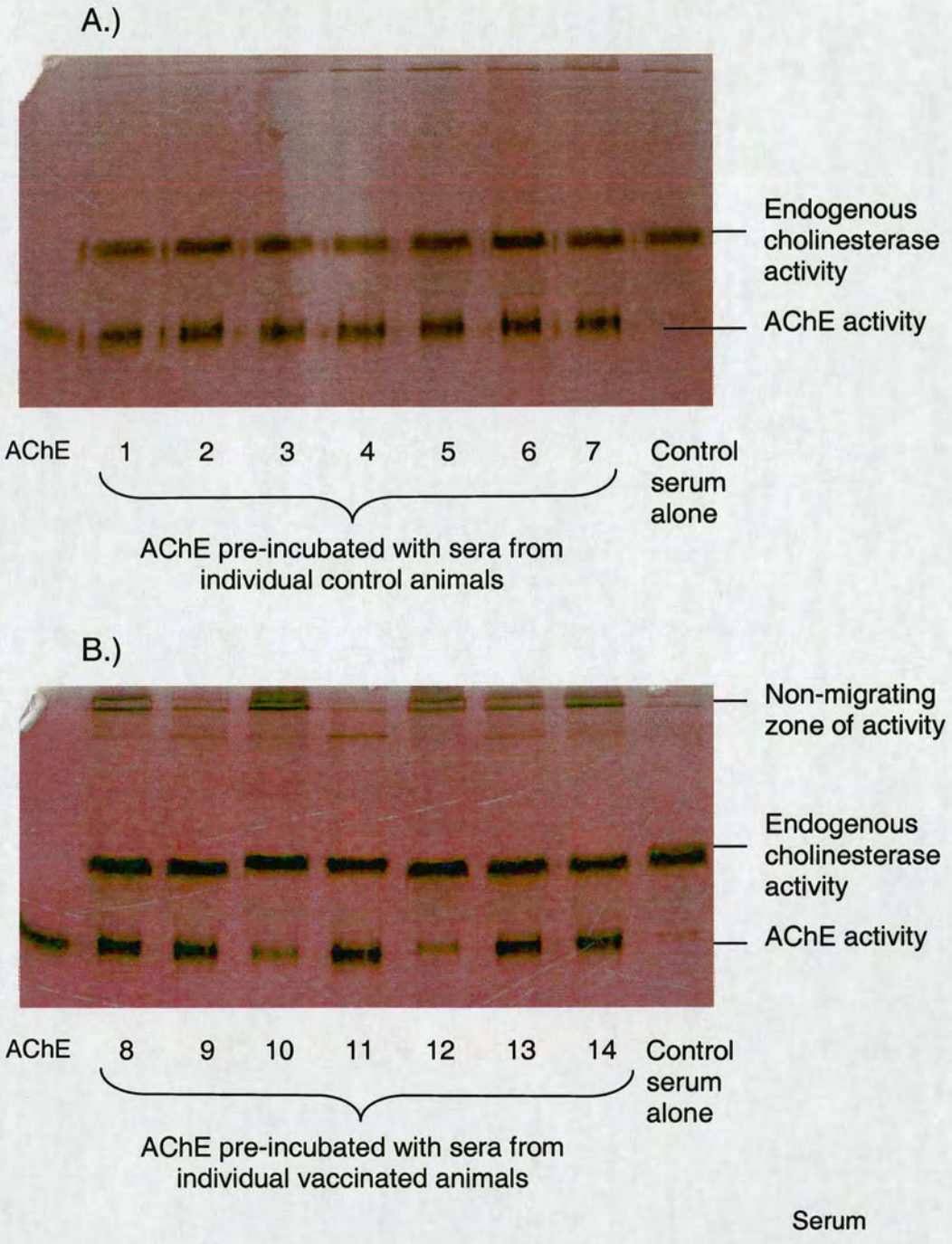


Figure 6.6. The effect of serum antibody on acetylcholinesterase activity. Serum from individual A.) Control (unvaccinated) and B.) AChE vaccinated animals was incubated with recombinant AChE, separated on Native PAGE gels and stained for cholinesterase activity. The activity associated with the recombinant AChE was reduced following incubation with serum from some AChE vaccinated rats (lanes 10 and 12) and there was an increase in activity high on the gel indicating a slowly or non-migrating product.

6.3 Discussion

This chapter described the first in a series of trials that tested the efficacy of recombinant AChE and SOD of *N. brasiliensis* as protective antigens in the rat challenge model. In this trial, the immunogenicity of the recombinant proteins and the protection conferred by systemic (SC) immunisation were tested. Protection was measured by comparing the cumulative faecal egg outputs from the vaccinated groups with groups of non-immunised animals (Control), or animals given adjuvant alone (Adjuvant). The immunogenicity was assessed by measure of the antibody and mast cell protease response of the animals.

The FEC of the AChE vaccinated group was markedly reduced through the course of the infection compared with the control, indicating that a protective effect had been conferred. The FEC of the SOD vaccinated group showed no such reduction and was slightly elevated compared with the control. These apparent differences in egg output were matched by differences in the specific antibody response generated against the immunising antigen between the groups. The AChE group showed significant increases in specific antibody isotypes over the controls while the SOD specific antibody levels were not significantly different from the control. The AChE vaccinated group also differed in the mast cell protease (RMCP II) response it expressed. Antibody from the serum of AChE vaccinated animals was shown to inhibit the migration of recombinant AChE through a native PAGE gel.

The cumulative FEC from the AChE vaccinated group was reduced by 48% compared with the control, indicating that a level of protection had been conferred. This finding agrees with the results from previous vaccination experiments in which immunisation with AChE enriched fractions provoked protective immune responses against *D. viviparus* (McKeand *et al.*, 1995a) and a mixed nematode infection including *T. colubriformis* (Griffiths and Pritchard, 1994). The apparent level of protection is similar to that seen in these earlier experiments, with approximately 50% protection conferred against *D. viviparus* infection in Guinea pigs (McKeand *et al.*, 1995a) and 30% protection against a mixed nematode infection (Griffiths and Pritchard, 1994).

The level of protection demonstrated was lower than that observed in a previous SC vaccination trial against *N. brasiliensis* in rats using an adult somatic extract as antigen (Murray *et al.*, 1979). In this trial the number of adult worms was reduced by 77% with a corresponding reduction in egg output. However, the trial was performed using large amounts (up to 10 mg) of a crude native antigen preparation containing a complex mixture of proteins and therefore might not be comparable to the trial described here.

The present data did not indicate that the differences in FEC from the control were statistically significant. This is most likely due to the high variability, a common problem with FEC data (Mes, 2003). Although there was likely to be a real effect, an increase in statistical power would be needed to resolve it to statistical significance (J. Sales, BioSS, pers. comm.). This could be achieved by an increase in the sample number. During this trial it was only possible to sample each cage rather than each individual animal (due to constraints of housing and welfare defined by the local Home Office Inspector). The sample size was therefore reduced from 7 to 3, reducing the power of the analysis. This trial was repeated in work described in Chapter 8 with the same result. As detailed in Chapter 8, combining the data from both trials allowed more powerful statistical analysis and showed that the effect was significant.

AChE-specific IgG titres were significantly increased in the serum of the AChE vaccinated group compared with the Control and Adjuvant groups. Both IgG2a and IgG1 were elevated by similar amounts indicative of a Th2 response to vaccination (Binder *et al.*, 1995). An increase in IgG, without necessarily a particular IgG2a or IgG1 (Th-1 / -2) bias was expected with this vaccination regime (Harlow and Lane, 1988; Cox and Coulter, 1997). That a particular IgG isotype is the effector of protection cannot be excluded because antibodies can have differing avidity for an antigen and this can be associated with whether or not a protective response is observed (Mulcahy *et al.*, 1998).

AChE vaccination also resulted in an increased serum IgA titre. IgA is a secretory antibody isotype that is active at mucosal sites (Miller, 1984), however no antibody was detected in the mucosa of any of the animals during this trial. As the major source of secretory IgA is the serum (Jackson *et al.*, 1978), the absence of

antibody in the mucosa indicates that no secretory / mucosal IgA response was generated in this instance. The lack of detectable antibody in the mucosa might also reflect a reduction in worm-mediated damage such that antibody was not transported across the mucosa from the circulation.

The titre of AChE-specific IgE in the AChE and Adjuvant groups was also significantly increased compared with the control. This antibody isotype is involved in hypersensitivity and inflammatory type responses indicating that such a response might be provoked in these animals. That AChE is antigenic for specific IgE during *N. brasiliensis* infection has previously been demonstrated (Nakazawa *et al.*, 1995). Elevated IgE levels support the view that the vaccination regime stimulated a Th-2 biased response.

The change in the activity profile of AChE when incubated with vaccinated serum does suggest a role of antibody in protection (Figure 6.6). The decrease in activity associated with the recombinant AChE isoform and the increase in activity higher in the gel compared with the control can be explained by antibody binding the AChE and forming a complex, which retains enzyme activity and migrates only slowly into the gel. This effect has previously been demonstrated in serum from *T. colubriformis* infected sheep and *D. viviparous* infections of guinea pigs, when the antibody isotype was identified as IgG (Rothwell *et al.*, 1976; McKeand *et al.*, 1995a). Such a mechanism *in vivo,* while not inhibiting the activity of AChE, might prevent its diffusion / dissemination and thus prevent its biological function in protecting the worm.

The level of RMCP II in the serum of the AChE vaccinated group was significantly lower than the control whilst that in the mucosa was significantly higher. These differences may indicate reduced mast cell activation and resultant RMCP II release. This scenario would cause the lower serum level with the increased mucosal level indicating the retention of RMCP II by mucosal mast cells. Another possibility is that worm damage to the mucosa was reduced in the AChE vaccinated group with the result that RMCP II did not leak into the systemic circulation to the same degree as in the controls. The FEC of the SOD vaccinated group showed no significant reduction and, indeed, was slightly elevated compared with the control, indicating that no protective effect had been conferred. SOD-specific antibody titres

were low with no significant differences between, SOD vaccinated, Control and Adjuvant groups. This indicates that the SOD may be only weakly immunogenic and therefore does not provoke a strong immune response. Strategies to overcome this might be to couple the recombinant SOD to a strong immunogen, denature the protein or to add groups such as dinitrophenol or arsenate to the protein (Harlow and Lane, 1988).

Another reason for the lack of response might be that the SOD protein was not recognised by the major histocompatibility complex (MHC) class 2 proteins expressed by this strain of rat. Genetic restriction of the MHC class 2 receptors expressed would lead to the lack of response by preventing antigen presentation to T-cells by antigen presenting cells (Harlow and Lane, 1988). MHC restriction of antigen recognition during rodent infection with helminth parasites is well known (Kennedy *et al.*, 1991; McKeand *et al.*, 1994a) and restricted recognition of ES antigens during *N. brasiliensis* infection has also been reported (Kennedy *et al.*, 1990). In this latter report the authors suggested that,

> "Immune selection might have resulted in the evolution of biochemically active exoantigens [ES enzymes] which are of poor immunogenicity or only subject to immune recognition in certain members of the definitive host species," (Kennedy *et al.*, 1990).

The hypothesis that MHC - class 2 protein restriction of the recognition of the recombinant SOD is responsible for the lack of immune response observed could be tested by immunising a different strain of rat in the same manner to see whether this increased response.

The level of RMCP II in either compartment of the SOD vaccinated animals also did not significantly vary from the level seen in the control. RMCP II levels in the serum and mucosa of Control animals were somewhat lower than the levels seen during the acute challenge infection at this time-point (Chapter 3). However, the lower level of infection used probably accounts for this difference. Protease levels were similar in the Adjuvant group in the serum but in the mucosa were elevated above the Control. A very large standard error in the mucosa results may account for this result and the Adjuvant group might, therefore, conform more closely to the pattern of high serum and lower mucosal levels seen in the control.

Pathogenic effects occurred in two individuals in the SOD immunised group during the course of vaccination. While these effects may have been due to some other cause than the vaccinating doses, the adjuvant Quil A is known to have some toxic side effects such as haemolysis due to its detergent properties (Estrada *et al.*, 2000). This may have been the cause of the side effects in rats although the absence of effects in both adjuvant control and AChE vaccinated groups, both receiving the same dose of Quil A, would appear to contradict this or suggest that only certain individuals might be affected. The SOD itself may have produced the effects seen, however the symptoms were not those of anaphylaxis and the low response to SOD seen in antibody levels would indicate only weak immunogenicity. This would appear to rule out an auto-immune or hypersensitivity reaction and another mechanism of pathology would be needed before a SOD-based explanation of the effects could be confirmed.

In conclusion, the results of this trial indicate that protection against *N. brasiliensis* infection may be conferred by vaccination with recombinant worm AChE, but no protective effect was seen with SOD vaccination. The protection in the AChE vaccinated group was associated with an increase in AChE-specific total IgG compared with the controls. Elevated levels of IgG2a and IgG1 isotype, in association with marked increases in serum IgE are indicative of a Th-2 response in vaccinated rats. No significant increase and no significant difference were seen in the SOD-specific antibody levels between control and SOD vaccinated animals. Differences in RMCP II level between the AChE group and the SOD and control groups might indicate reduced mast cell activation.

Chapter 7 - The effect of immunisation using *Nippostrongylus brasiliensis* recombinant acetylcholinesterase via the intra-peritoneal route in Wistar rats and recombinant superoxide dismutase via the sub-cutaneous route in Sprague–Dawley rats

7.1 Introduction

Chapter 6 described a protection trial in which rats immunised with a single recombinant form of a *Nippostrongylus brasiliensis* AChE were partially protected against challenge infection, as judged by a reduction in faecal egg output compared with the control. In that trial the antigen was administered systemically with Quil A as adjuvant. The immune response to vaccination was characterised by increases in IgG1, IgG2a and IgE isotypes indicative of a Th-2 response.

Sera (presumably serum antibody), from vaccinated rats, was also demonstrated to inhibit the migration of the recombinant enzyme on a PAGE gel stained for enzyme activity. Such an effect has been noted previously with sheep, which had been infected with *T. colubriformis*, producing antibodies that bind to the worm AChE (Rothwell and Merritt, 1974). Guinea pigs vaccinated with an AChE-enriched fraction from adult *D. viviparus* were partially protected against subsequent challenge infection, and IgG from their serum inhibited AChE activity (McKeand *et al.*, 1995b). In the study described in Chapter 6, there was, however, no detectable local antibody response in the mucosa. It is possible that a vaccination regime which is designed to stimulate local mucosal immune responses may enhance the level of protection observed in the previous experiment (Emery *et al.*, 1993).

As discussed in Chapter 1, worm expulsion from the GI tract has been associated with both systemic and local antibody and immune cell responses in the mucosa, with mast cell activation being identified as a key event (Balic *et al.*, 2000). In the case of *N. brasiliensis*, antibody responses have previously been described during infection (Ogilvie and Jones, 1971) and infection is known to induce large

specific and non-specific IgE responses (Jarrett and Haig, 1976; Yamada *et al.*, 1991). Mastocytosis in the intestinal mucosa with subsequent mast cell degranulation and release of mast cell proteases are observed (Huntley *et al.*, 1993). Goblet cell hyperplasia also occurs and both the amount of mucus increases and glycosylation of mucus secreted changes during expulsion (Karlsson *et al.*, 2000). Worms can become trapped in mucus and the latter contains local antibodies including IgG and IgA (Wedrychowicz *et al.*, 1983), which have been ascribed roles in worm expulsion (Balic et al, 2000). This highlights the possibility that successful vaccination might require similar effector immune responses.

Use of different adjuvants administered with vaccines may influence the T-cell (Th-1 / -2) bias of the primed immune response (Cox and Coulter, 1997). The Th balance of the immune response may influence the outcome of infection, as protective responses against nematodes are associated with Th-2 responses (Urban *et al.*, 1992). Different adjuvants administered with vaccine antigens have previously been shown to influence the protection conferred by vaccination against nematodes (Jacobs *et al.*, 1999).

In the previous vaccination experiment, recombinant SOD and AChE antigens were administered systemically with Quil A, an adjuvant considered to promote a high circulating antibody titre and Th-1 and -2 responses (Cox and Coulter, 1997). The outcome of the experiment indicated that AChE might provoke a protective immune response as indicated by a marked reduction in FEC in vaccinates compared to controls. The antibody response was a systemic event with no detectable antibody in the small intestine mucosa.

The experiment described in this chapter was therefore designed to target the antigen to provoke a mucosal immune response. One way to achieve this is to present the antigen by the IP route. The peritoneum or gut cavity contains many mucosal surfaces and administration by this route is considered to enhance presentation of the antigen to the mucosa as well as boosting a large systemic immune response in rodents (Harlow and Lane, 1988). By immunising in this way it was hoped to provoke a local, mucosal response against the parasite. Antigen was presented in combination with aluminium hydroxide (alum) adjuvant. This adjuvant boosts immune responses with a bias towards Th-2 response (Cox and Coulter, 1997). As

the previous vaccination experiment provoked an antibody response that showed no clear Th polarisation it was considered worthwhile to evaluate if this adjuvant might improve the level of protection observed by stimulating a mucosal response with a Th-2 bias.

In the previous experiment immunisation with SOD did not lead to a high SOD specific antibody titre in the vaccinated animals possibly because the protein is not particularly immunogenic. The lack of antibody response may also have been due to immune restriction. Mammals, including rodents, are able to express only a finite number of major MHC receptors, which allow them to recognise foreign antigens. MHC-restricted antibody recognition to helminth parasites has been previously reported (Kennedy *et al.*, 1991; McKeand *et al.*, 1994a).

To test the hypothesis that the poor antibody response was due to the genetic background of the Wistar rats, a separate strain of rat, Sprague–Dawley, was inoculated in the same manner as before. This experiment was designed to determine whether the strain of rat could influence the outcome of immunisation and generate an increased antibody response. The Sprague–Dawley strain has previously been used in *N. brasiliensis* challenge experiments. The infection cycle has been well characterised in this strain and conforms to the pattern seen previously in the Wistar strain of rats (reviewed; Ogilvie and Jones, 1971).

7.2 Results

7.2.1 Observations

In general the immunisations were well tolerated with no adverse reaction. However, a single animal in the group immunised SC with AChE in combination with Quil A had to be put down after developing nasal bleeding following the second immunisation. A subsequent post-mortem was unable to give a definitive cause of pathology other than to confirm that there was bleeding from the respiratory system.

7.2.2 Pre-challenge serum antibody level

One week prior to challenge serum was collected by tail bleed and the total IgG titre specific for AChE or SOD was measured by ELISA as described in Chapter 2.7.

The antigen specific IgG titre of the group of animals immunised with AChE / SC was significantly increased compared with the control, with a titre of 490 (P=0.006). Delivery of AChE via the IP route give a much higher mean IgG titre of 36,000 which was, again, significantly higher than the controls (P=0.0015).

The group of Wistar rats immunised SC with SOD indicated a specific IgG titre of 127. This titre did not, however, represent a significant increase compared with the Wistar controls. The SOD specific antibody titre of the Sprague-Dawley rats also did not differ significantly from that of the Sprague-Dawley controls.

7.2.3 Faecal egg counts

Faeces were sampled from 5 DPI and FEC performed as described in Chapter 2.1. Results of these FEC are displayed in Figure 7.1, panels A - D.

Results from all the groups, including the control, were lower than had been seen in earlier experiments (Chapter 3 and 6). The Wistar control group peaked at less than 50 eggs / g faeces on day 7 after infection with the Sprague-Dawley control peaking at less than 100. The AChE / SC peaked at less than 100 and AChE / IP at less than 25. Both SOD vaccinated groups peaked between 50 and 100 eggs /g faeces. In addition there was high variability in the FEC from the different samples within each group. In some samples there was a minimal (or zero) egg output indicating that a patent infection had not been established while other cages in the same group had a higher egg output.

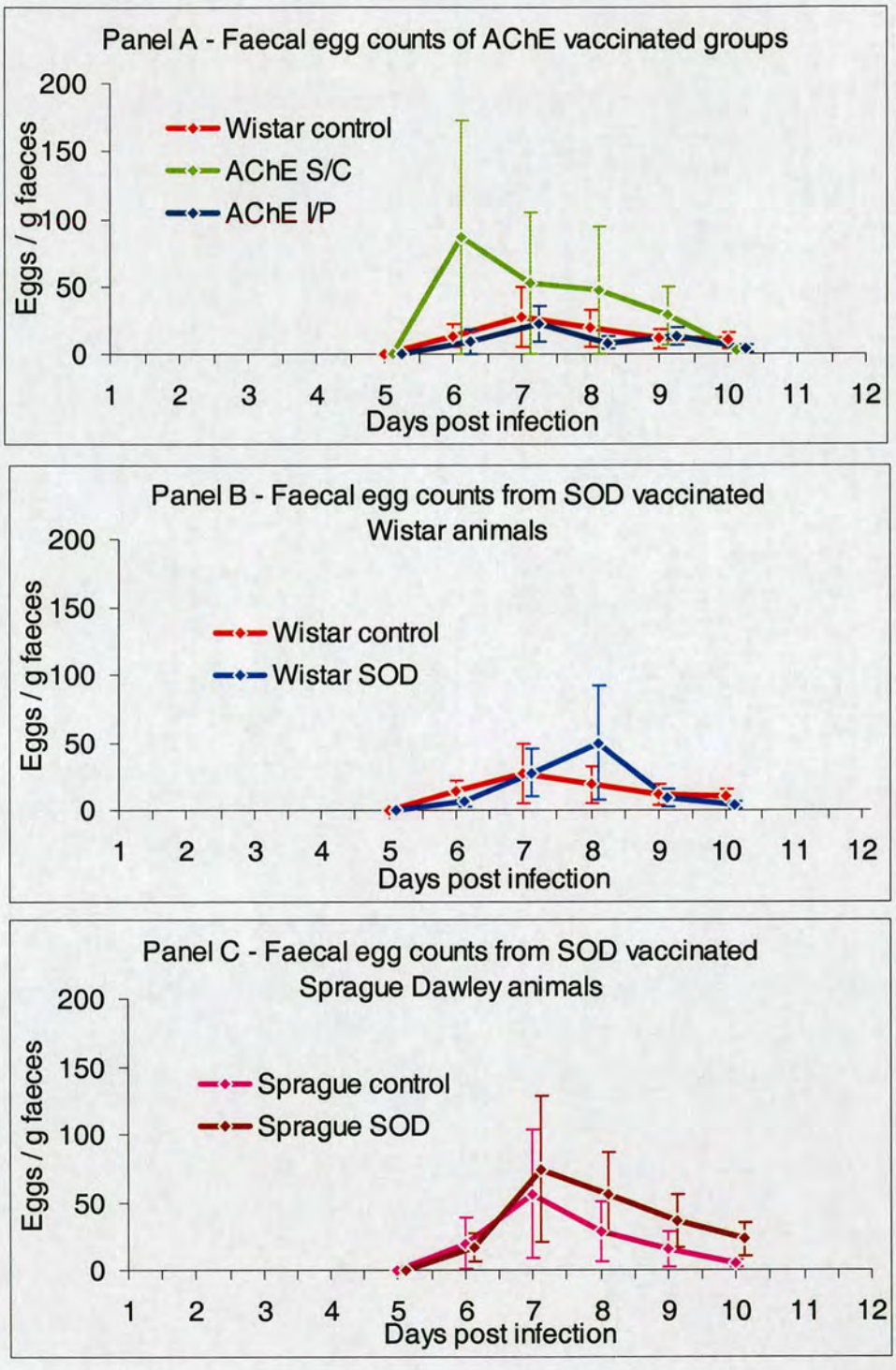


Figure 7.1. The faecal egg count from Control (unvaccinated), SOD and AChE vaccinated Sprague-Dawley and Wistar rats for 11 days following challenge infection with 25 *Nippostrongylus brasiliensis* L3 larvae. The data represent the mean of faecal egg counts performed on three pooled samples from each group at each timepoint (+/- the standard error). The faecal egg outputs varied dramatically from those previously observed.

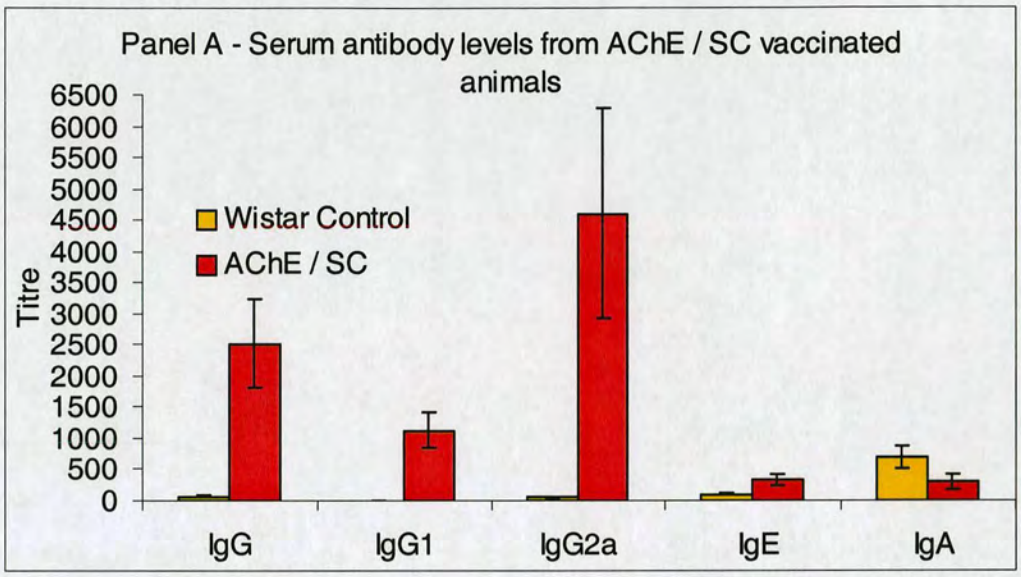
7.2.4 Post challenge serum antibody levels

Serum antibody levels were measured by ELISA following necropsy at day 11 post infection, as described in Chapter 2.7 and the results of these assays are demonstrated in Figure 7.2, panel A and B.

The total IgG titre, as well as both IgG1 and IgG2a subtypes, from the AChE / SC vaccinated animals were significantly elevated compared with the control group ($P<0.003$). The IgG2a component appeared more prominent at 4,600 compared with an IgG1 titre of 1,100 (Figure 7.2, panel A). IgE and IgA isotype titres displayed no significant differences from the control in this group.

Serum antibody levels from the AChE / IP vaccinated group are displayed in Figure 7.2, panel B. In general AChE specific antibody responses in this group were much larger than those seen previously. The total IgG titre showed the largest increases at over 40,000. Of this total the specific IgG1 titre was 16,000 and the specific IgG2a titre, 26,000. Increased titres of AChE specific IgE (20,000) and IgA (15,000) were also observed. The increases in AChE specific antibody titre in the serum of the AChE / IP vaccinated group when compared with the control group were all statistically significant ($P\leq0.02$).

The SOD-specific antibody titre of all antibody isotypes measured in the serum from SOD immunised Wistar and Sprague-Dawley rats was not increased or significantly different from the respective controls (data not shown).



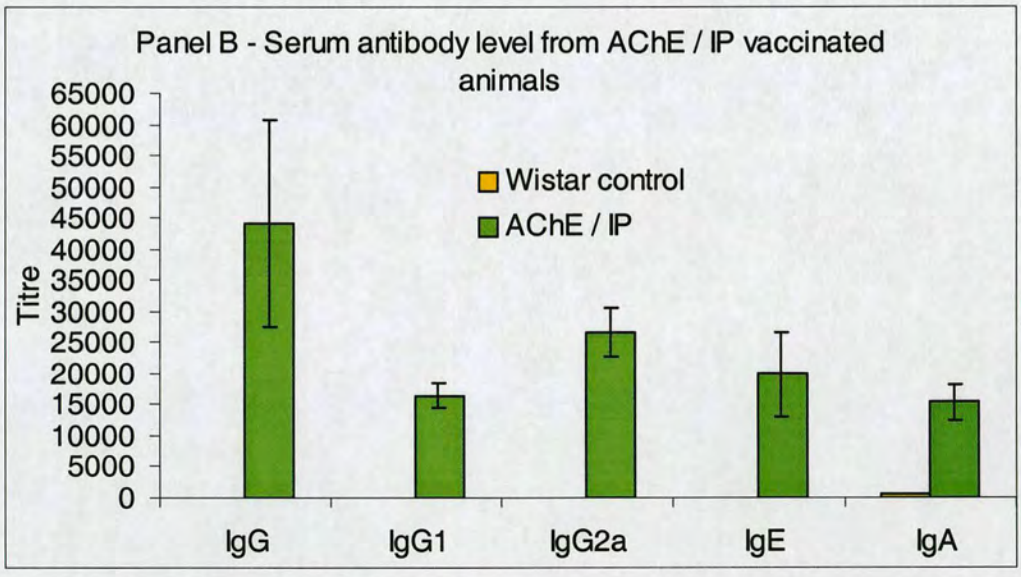


Figure 7.2. The AChE specific serum antibody response of Wistar rats vaccinated with; A.) AChE / SC and B.) AChE / IP compared with Control (unvaccinated) animals at 11 days following challenge with 25 *Nippostrongylus brasiliensis* L3. The data represent the mean antibody titre of the 7 animals in each vaccination group measured by ELISA (+/- the standard error). Some differences in the levels of specific antibody were observed between the groups.

7.2.5 Mucosal antibody levels

Mucosal antibody levels were detectable in AChE vaccinated animals and were measured by ELISA, as described in Chapter 2.7. Antibody isotype titres from the Wistar control group and the AChE vaccinated groups are displayed in Figure 7.3, panel A and B.

As demonstrated in Figure 7.3, panel A, the titres of AChE-specific antibody isotypes IgG1, IgA and IgE measured from the mucosa of the AChE / SC vaccinated animals did not differ significantly from the controls. Small but statistically significant increases in total IgG and IgG2a over the controls were observed ($P \leq 0.01$).

The results from the group vaccinated with AChE / IP are displayed in Figure 7.3, panel B. AChE-specific antibody titres were increased in this group compared with the control. The largest increase was seen in the titre of IgG, which had a titre of 750. Titres of IgG1 and IgG2a were both elevated, with IgG1 titres of 105 and IgG2a titres of 210. Specific IgA and IgE titre were also both increased over control levels with titres of 175. These increases in the specific antibody titre were statistically significant when compared with the titre of specific antibody from Wistar controls ($P \leq 0.03$).

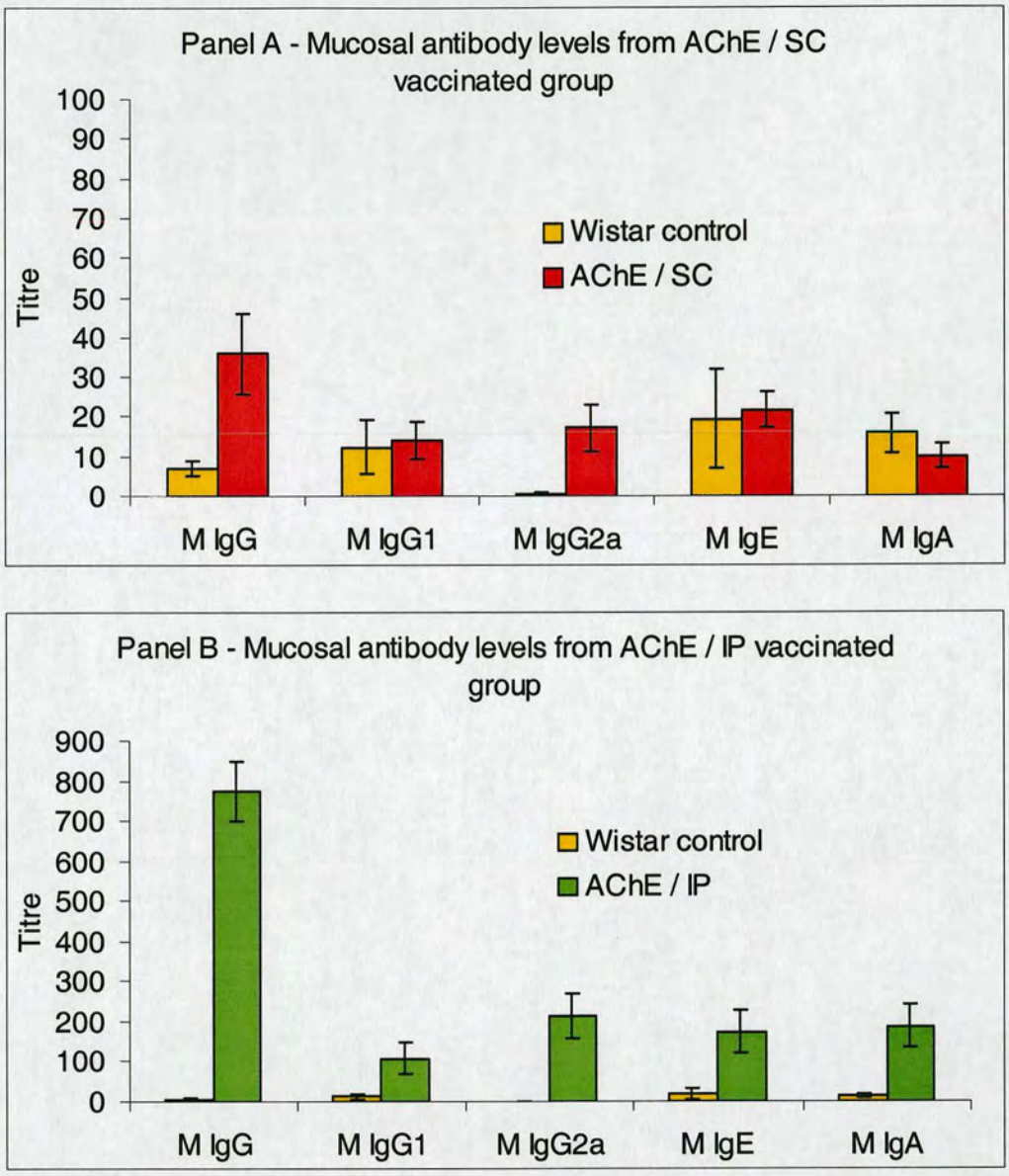


Figure 7.3. The AChE specific mucosal antibody response of Wistar rats vaccinated with; A.) AChE / SC and B.) AChE / IP compared with Control (unvaccinated) animals at 11 days following challenge with 25 *Nippostrongylus brasiliensis* L3. The data represent the mean antibody titre of the 7 animals in each vaccination group measured by ELISA (+/- the standard error). Some differences in the levels of specific antibody were observed between the groups.

7.2.6 Serum and mucosa mast cell protease levels

RMCP II levels were measured from the serum and mucosa of the Wistar control and the AChE vaccinated groups by ELISA following necropsy at day 11 post infection, as described in Chapter 2.7. The results of these ELISAs are displayed in Figure 7.4.

The serum levels of RMCP II were low in each of the vaccinated groups and in the control group, ranging between 150 and 300 ng/ml. No statistically significant differences were demonstrated between levels of RMCP II in the serum of the vaccinated and control animals. The serum levels of RMCP II, demonstrated in these three groups of animals, were similar to those demonstrated previously in uninfected control animals (Chapter 3).

The mean RMCP II level in the mucosa of the control group animals was elevated compared with normal uninfected rats, at 684 ng/ml. The level of RMCP II in the mucosa of the AChE / SC vaccinated animals was significantly less than that demonstrated in the infection control, at 200 ng/ml (P=0.05). The AChE / IP vaccinated groups had a mean mucosal RMCP II level of 300 ng/ml, which was not significantly different from that of the infection control group.

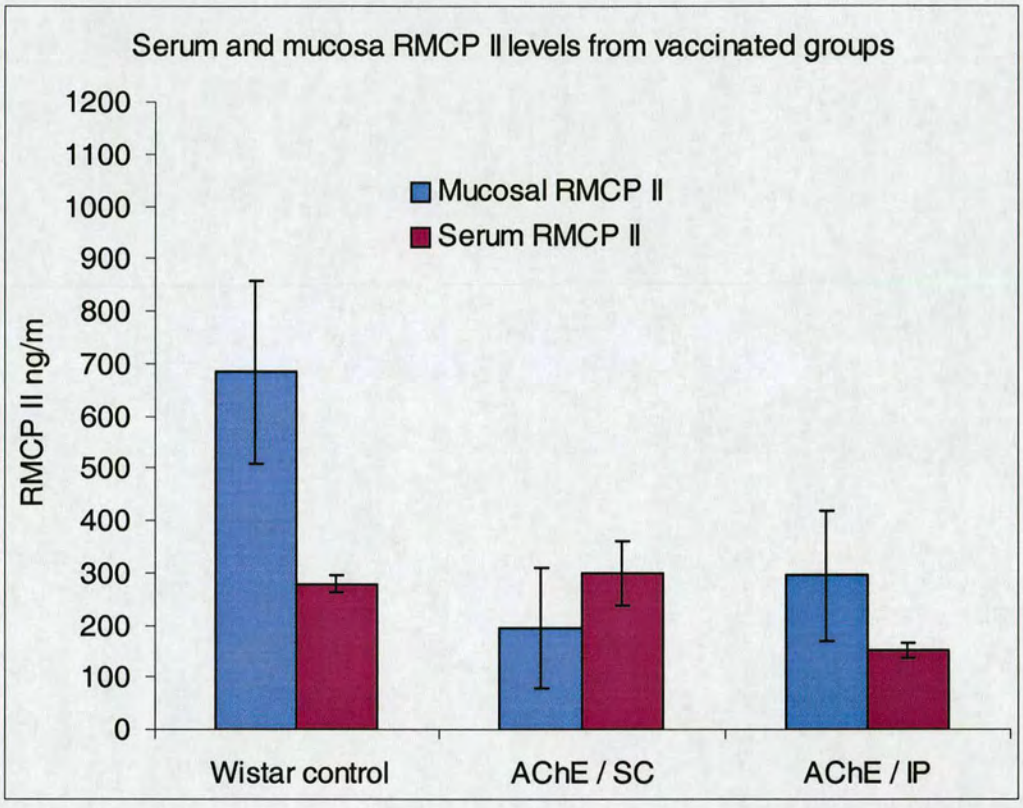


Figure 7.4. The mast cell protease response in the mucosa and serum of Wistar rats vaccinated with AChE, and unvaccinated Controls, at 11 days following challenge with 25 *Nippostrongylus brasiliensis* L3. The data represent the mean protease level of the 7 animals in each vaccination group measured by ELISA (+/- the standard error). Some differences in the levels of RMCP II were observed between the groups.

7.2.7 The effect of antibody on acetylcholinesterase activity

The effect of antibody on the activity of recombinant AChE was investigated by incubating recombinant AChE with serum from control and vaccinated animals as described in Chapter 2.5.5. Following this incubation the samples were separated on native PAGE gels and stained for esterase activity. Gels containing recombinant AChE incubated with serum from control and AChE / IP vaccinated animals are displayed in Figure 7.5, panel A and B.

The pattern, observed previously with control serum, of two major bands of activity corresponding to endogenous activity and recombinant AChE activity, is shown in Figure 7.5, panel A. The AChE / IP vaccinated serum (Figure 7.5, panel B) indicated bands of activity corresponding to recombinant AChE that were much weaker than the control (lanes 8, 9 and 13), and in some cases absent completely (lanes 10, 11, 12 and 14). There were also no bands of activity higher up on the gel, previously observed when recombinant AChE was incubated with serum from AChE / SC vaccinated animals and indicative of very slowly migrating products (Chapter 6.2.8).

An attempt was made to replicate this experiment with soluble homogenate containing antibody from the mucosa of the AChE / IP vaccinated animals. This however proved impractical due to the presence in the mucosal homogenates of several bands of endogenous cholinesterase activity that made it impossible to distinguish recombinant AChE and antibody bound AChE banding patterns (data not shown).

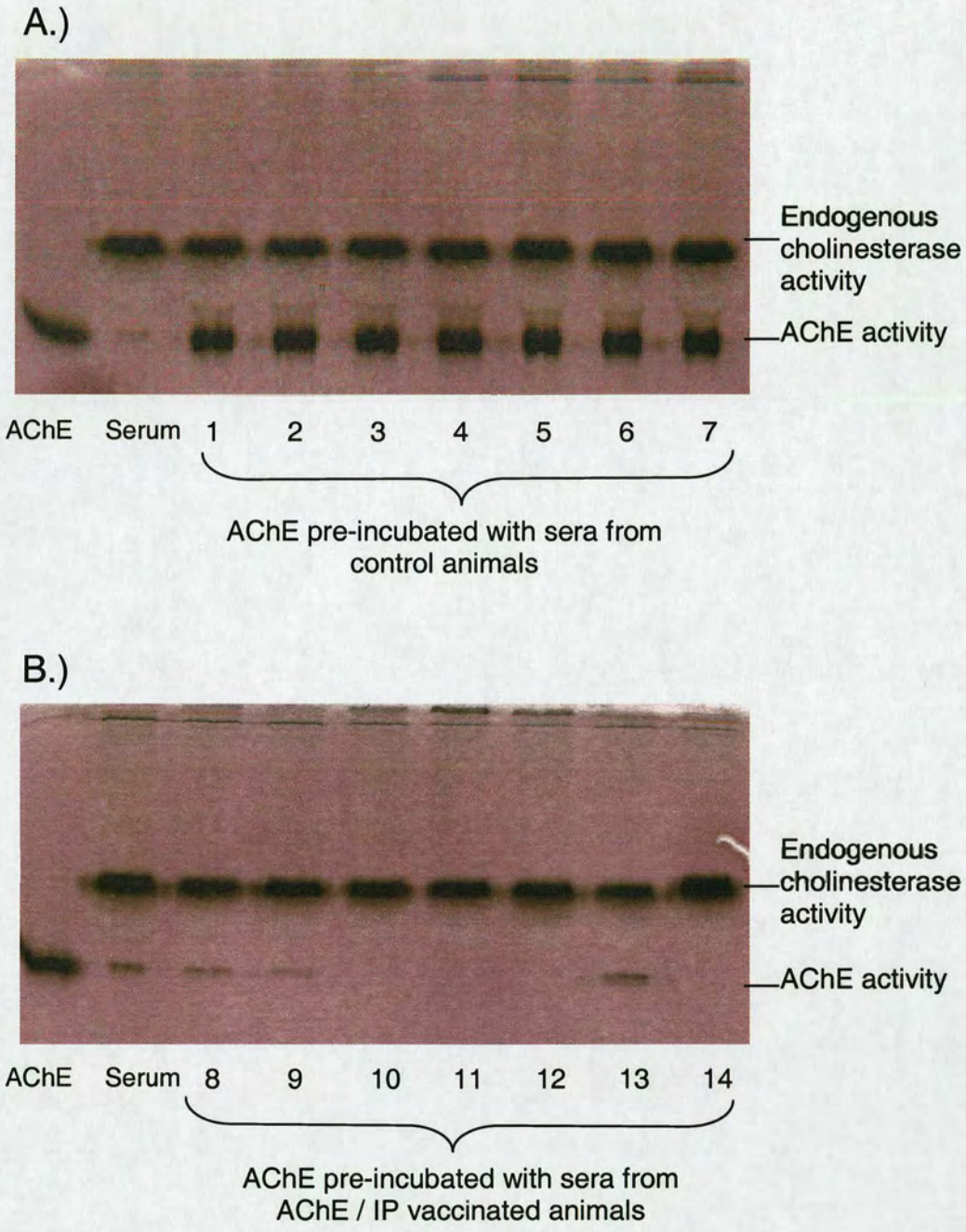


Figure 7.5. The effect of serum antibody on the activity of recombinant acetylcholinesterase. Serum from individual A.) Control (unvaccinated) and B.) AChE / IP vaccinated Wistar rats was incubated with recombinant AChE, separated on Native PAGE gels and stained for cholinesterase activity. The activity of the recombinant AChE was dramatically reduced by the addition of the AChE / IP serum (lanes 8 – 14).

7.3 Discussion

The experiment outlined in this chapter had three aims. First to confirm the outcome of the trial described in Chapter 6. Second, to investigate whether IP antigen delivery would result in a detectable humoral immune response in the intestinal mucosa and enhanced protection. Third, to investigate if the poor immunogenicity of SOD in Wistar rats, noted in Chapter 6, could be circumvented by using a different rat strain.

The results of the challenge infection from the control groups indicated that the establishment of infection was poor when compared to previous experiments (Chapters 3 and 6). In previous experiments, faecal egg output peaked at approximately 200 eggs per gram faeces but here, peak output was consistently less than 100 eggs per gram faeces and the data were more variable (Figure 7.1). In fact, faecal egg output from the controls was often lower than that from vaccinates. Consequently it was impossible to ascertain the level of protection, if any, that was conferred by vaccination with any of the antigens. Despite this problem, it was notable that the lowest egg output was recorded from the AChE / IP group with this tending to be lower than any other group in the experiment. In a previous vaccination experiment performed in this system IP vaccination produced the highest level of protection using a somatic extract antigen when compared with SC and oral immunisations (Murray *et al.*, 1979).

The reason for this failure to produce a viable infection is thought to be the age of the faecal cultures from which the infection doses were harvested (D Knox, pers. comm.). As these cultures were several months old the viability of the larvae may have been reduced to a level such that their infective capacity was impaired. Measures to prevent a repeat of this possible failure in future infection were implemented. These included the preparation of fresh larval cultures and the use of tracer animals prior to challenge of vaccinated groups to check the infective capacity of the larvae. These measures are discussed in greater detail in Chapter 8.

Strong immune responses, as judged by increases in specific antibody titres, were again generated by vaccination with the recombinant AChE. SC immunisation resulted in higher total serum IgG titres than those observed in the previous trial (2,500 vs. 400) with IgE and IgA titres being lower. Vaccination via the IP route

produced the highest serum antibody titres with all isotypes having titres higher than 10,000. In addition, increased levels of all specific antibody isotypes were detected in the mucosa of IP vaccinated rats, while smaller increases in only two mucosal antibody isotypes were seen in the SC vaccinated animals. These data confirm that IP immunisation does stimulate a higher systemic and mucosal response in comparison to SC delivery as expected (Harlow and Lane, 1988). Unfortunately, due to the problems with the infectivity of the challenge dose of *Nippostrongylus* larvae it proved impossible to determine any increased level of protection afforded by this increased immune response or if the mucosal response would enhance protection.

The observed differences in the antibody response of the SC groups between trial results might be due to the failure of the infecting dose of worms to establish a similar level of infection in each of the trials. If this were the case then the previously observed IgE and IgA responses would have been a result of *N. brasiliensis* infection and not a result of the vaccination alone (Chapter 6). This further suggests that the primary immune response generated by the vaccination alone might be an IgG response particularly of the IgG2a isotype. Native AChE has been demonstrated previously to be immunogenic for the stimulation of IgE during *N. brasiliensis* infection (Nakazawa *et al.*, 1995). *N. brasiliensis* infections are also well known to stimulate IgG1, IgA and especially IgE responses (Jones *et al.*, 1970; Jarrett and Haig, 1976; Jarrett and Bazin, 1977). *Nippostrongylus* antigens have also been demonstrated to possess an adjuvant effect for Th-2 responses, including IgE and IgG1 production driven by IL-4 (Holland *et al.*, 2000)

The effect of antibody binding on AChE activity was determined using sera from the AChE / IP vaccinated animals. Sera from all immunised rats inhibited recombinant AChE activity (Figure 7.5), an effect which may be due to antibody-mediated inhibition of enzyme activity by the antibody (Rothwell *et al.*, 1976; McKeand *et al.*, 1995a). Alternatively it might indicate a binding effect, such as that observed in the previous experiment (Chapter 6.2.8), but of increased efficacy. This effect may possibly prevent AChE from entering the gel, and enzyme activity is therefore not seen. An attempt to further characterise this effect by titrating the serum concentration to see if the profile changed to one similar to that of the AChE / SC vaccinated sera unfortunately did not produce a result due to the confounding effect

of endogenous esterases in the serum (data not shown). Either an inhibitory or increased binding effect might be due to the increased titres of antibody present in the serum of AChE / IP animals when compared with the AChE / SC vaccinated animals. The inhibitory effect of the serum on the AChE activity might also have been due to an increase in either the predominant IgG isotype, or of one of the other antibody isotypes (IgE or IgA). Purifying antibody subclasses for examining this effect could be achieved by column chromatography, but was beyond the scope of this project. Previous work on *D. viviparus* AChE indicated that a similar inhibitory effect of antibody was due mainly to the IgG isotype (McKeand *et al.*, 1995b).

An element of this experiment was designed to test the hypothesis that the lack of response of rats to vaccination with recombinant worm SOD may be due to genetic restriction of antigen recognition dictated by the strain of rat used in the previous trial (Chapter 6). Genetic restriction of MHC-antigen recognition during infection with helminth parasites is well known (Kennedy *et al.*, 1991; McKeand *et al.*, 1994a). Immunisation of a second strain of rat with recombinant SOD in combination with Quil A, using the same route of delivery (SC) did not result in an increased immune response to the antigen. Low responses were again observed in the Wistar strain indicating that the lack of effect might be due to low immunogenicity of the SOD protein rather than immune restriction. However, it is notable that sheep vaccinated with SOD from *H. contortus* did mount a vigorous antibody response and were partially protected against challenge infection (Liddell and Knox, 1998). These data suggest that SOD is immunogenic and that the problem here is likely to be due to restricted recognition dictated by the strain of rat used. This suggestion is further supported by the earlier observation that hyper-immune sera from different rats varied in their recognition of the recombinant SOD (Chapter 4).

The levels of RMCP II in the serum and mucosa of the vaccinated and control animals at the end of this trial differed from those observed previously (Chapter 3 and 6). Whereas in the previous trial elevated mucosal and serum levels were seen in the control, in this trial elevated mucosal levels were observed but serum levels were similar to those observed in uninfected rats. As mast cell accumulation and degranulation is strongly provoked during *N. brasiliensis* infection (Miller *et al.*, 1983b), this may be an indication of the reduced stimulation of mast cell aggregation

and degranulation induced by the lowered level of infection seen during this challenge. Indeed levels of RMCP II in the sera of both the control and vaccinated groups did not appear to differ from the level demonstrated previously in uninfected control animals (Chapter 3). This suggests that there was no detectable activation of mast cells at this time-point of the trial.

The amount of RMCP II in the mucosa of the AChE / SC and AChE / IP groups differed from the control group, with lower levels observed. The levels of both mucosal and serum RMCP II in these groups were similar to those seen in the AChE / SC group in the previous trial (Chapter 6). Once again the differences between control and vaccinated groups may have been conferred by the treatment, but with the differing results of the control groups between the two trials caused by the effect of the reduced infection level. The observed levels again suggest that vaccination with AChE had a significant effect, reducing recruitment and activation of mast cells during this infection. However, due to the differences in the infection level between the experiments, interpretation of such an effect during this second trial may be harder to explain. Any conclusions based on this trial may be flawed due to the confounding effects of the reduced infection level on mast cell protease levels.

In summary, it was concluded that vaccination with AChE and alum via the IP route did increase antibody titres in both the serum and the small intestinal mucosa compared with AChE and Quil A via the SC route. There was a corresponding increase in the inhibitory effect on the activity of the AChE enzyme linked to this increase in specific antibody titre. It was not possible to determine any effect that this improved antibody response might have on protection however.

It was also concluded that the low response of animals to SOD was due to the low immunogenicity of the protein and not immune restriction. As attempts to improve immunogenicity might be time consuming and unsuccessful, SOD was therefore not included in further vaccination trials but it was decided to concentrate effort on the more effective AChE antigen.

Chapter 8 – The effect of vaccination with recombinant *Nippostrongylus brasiliensis* acetylcholinesterase by the intra-nasal route

8.1 Introduction

In previous nematode vaccination trials in sheep, animals have generally been immunised systemically, producing a circulating antibody response (Emery *et al.*, 1993). Immunisation via the SC route produces a systemic antibody response in rodents (Harlow and Lane, 1988). A previous trial with recombinant *N. brasiliensis* AChE delivered via this route produced a significant AChE-specific antibody response in the serum and a 48% level of protection. This result however was not statistically significant due to experimental constraints (Chapter 6 and 7).

Immunisation via the IP route produces high circulating antibody titres in rats (Harlow and Lane, 1988) and also stimulates a mucosal immune response. Immunisation via this route was more effective in producing protection against *Nippostrongylus* infection than SC immunisation, using adult somatic extract antigen (Murray *et al.*, 1979).

The use of aluminium adjuvants also boosts antibody responses and may polarise the antibody response towards a Th-2 response (Nicklas, 1992; Cox and Coulter, 1997), of the kind thought to act against nematode parasites (Urban *et al.*, 1992; Finkelman *et al.*, 1997). In a previous experiment, immunisation via the IP route using alum adjuvant produced a higher antibody titre in the serum (Chapter 7). Using this regime, specific antibody was also detected in the intestinal mucosa, an important site in the protection against GI nematodes (Miller, 1984). Unfortunately, any protection conferred by these heightened antibody responses could not be assessed due to experimental difficulties (Chapter 7).

Stimulation of Th-2 immune responses (Akhiani, 1997) and protection against gut dwelling nematodes (Tsuji *et al.*, 2001; McGuire *et al.*, 2002) have been demonstrated by immunising via the intra-nasal (IN) route using cholera toxin (CT),

or its component β-subunit (CTB), as an adjuvant. CT and CTB are powerful mucosal adjuvants, which promote local as well as systemic immune responses (Elson and Ealding, 1984; Tochikubo *et al.*, 1998). Type 2 responses may also be preferentially stimulated by CT adjuvant (Akhiani, 1997). This route and choice of adjuvant may be useful for stimulating immunity in the intestinal mucosa as priming at one mucosal surface may stimulate a response at another via the common mucosal immune system. This site is important in the defence against GI nematodes and therefore stimulating responses in this tissue might be appropriate to elicit protective immunity (Miller, 1984).

The third vaccination trial was therefore designed to address these issues. Firstly to repeat the protection trial with SC and IP vaccination with AChE and secondly to measure the immune responses and assess the protective capacity of IN vaccination with the same antigen.

In order to overcome the problems faced in the previous vaccination trial, several measures were taken to ensure the viability of the infection doses prior to the challenge to the vaccinated animals. Fresh faecal cultures were prepared and timed to produce fresh, mature L3 larvae during the period when challenge would take place. The infective capacity of these larvae was also tested on several tracer animals one week prior to challenge of the vaccinated animals. FEC were measured to confirm that these infections produced similar levels to those demonstrated previously (Chapter 3).

8.2 Results

8.2.1 Observations

During this experiment there were no problems related to the immunisation such as were observed during the first two vaccination trials. Animals immunised with AChE / Quil A were closely monitored for associated pathology but none was detected.

With regard to the nasal immunisation regime, it was observed that the animals often exhaled (or sneezed out) a significant quantity of the immunising dose, either immediately on it being applied to the nares or after being inhaled for one breath. This tended to occur despite the mild anaesthesia under which the immunisations were applied to the animals. This observation led to a concern that animals may not have received a sufficient dose of the immunising antigen or that the response of individuals would vary markedly.

8.2.2 Pre-challenge serum antibody levels

Prior to challenge the vaccinated animals were bled and the serum levels of total IgG were measured by ELISA to assess the response to vaccination, as described previously (Chapter 2.8.2).

While each vaccinated group demonstrated an IgG response, the level of this response varied between each group (data not shown). The highest IgG titres were generated with IP delivery with alum (AChE / IP). Elevated titres, albeit of a lower magnitude, were observed following SC delivery with Quil A (AChE / SC) and, encouragingly, IN delivery with CT (AChE / IN).

8.2.3 Faecal egg counts

Faecal egg counts – Trial 3

FEC were performed on samples from tracer and vaccinated animals as described in Chapter 2.1.4 (Figure 8.1, panel A – C). The results from the tracer animals indicated that in this experiment the infective capacity of the L3 larvae, as judged by FEC produced, was similar to previous experiments (data not shown). This was confirmed by the FEC from the vaccination control group, which conformed closely to the levels of egg output seen in earlier successful infection experiments (Chapters 3 and 6).

Both the SC and IN vaccinated groups (Figure 8.1, Panels A and C) demonstrated reduced FEC compared to the controls. These corresponded to a 23% and a 38% reduction in the cumulative egg output respectively. In the SC group, the effect was particularly evident during the earlier stages of infection. These reductions were not statistically significant.

Although early egg output was reduced in the IP group, this was due to a lag in egg output that was offset by a longer plateau of peak output compared to the controls. This resulted in the IP group producing a similar cumulative egg output to the controls (Figure 8.1, Panel B).

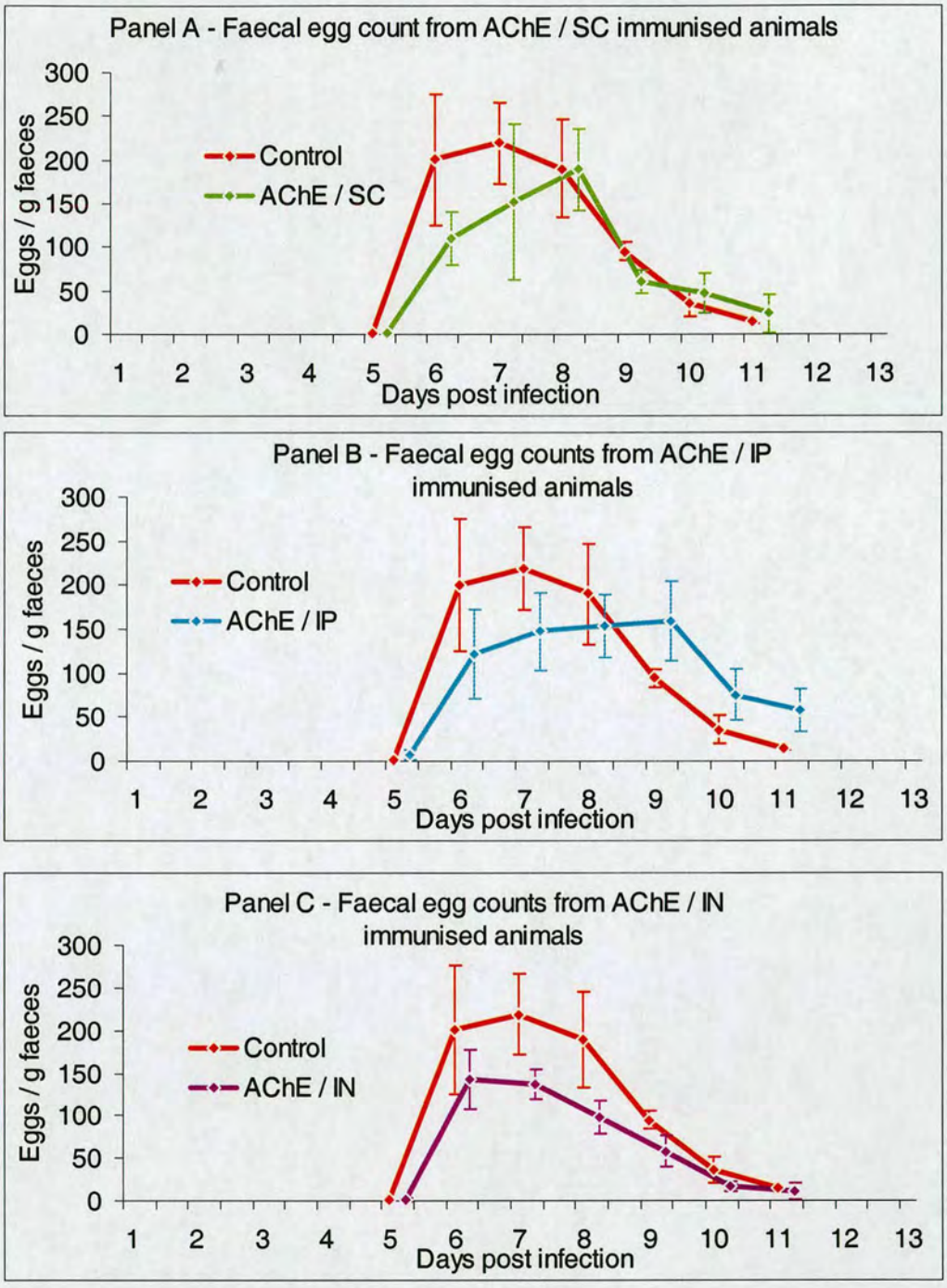


Figure 8.1. The faecal egg output from AChE vaccinated and unvaccinated Control Wistar rats over 11 days following challenge infection with 25 *Nippostrongylus brasiliensis* L3 larvae. Data represent the mean of counts performed on three pooled samples collected from each group of animals (+/- the standard error). Vaccination with AChE / SC and AChE / IN reduced egg counts compared with the control.

Statistical analysis of combined Trial 1 and 3 faecal egg count data

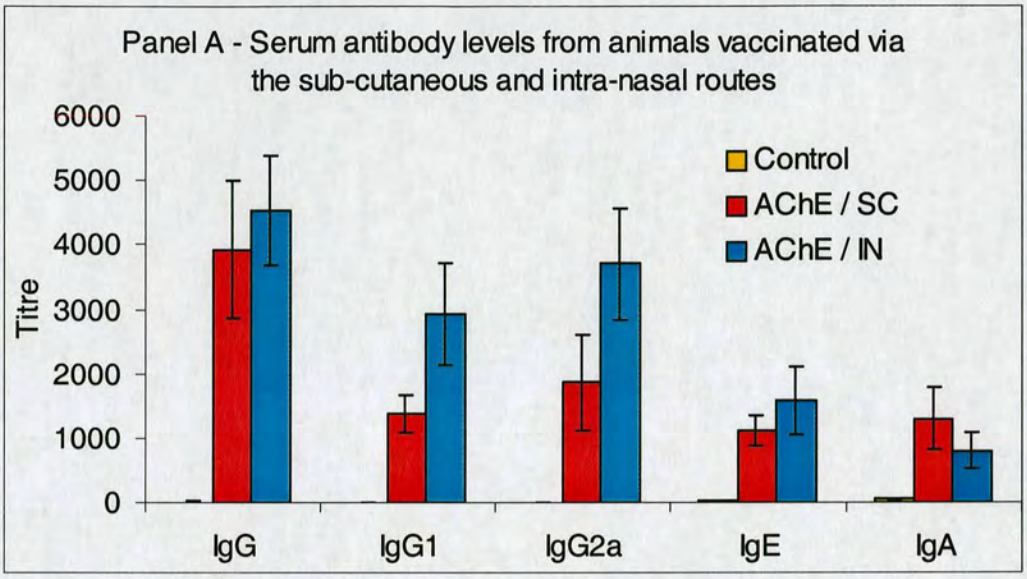
In order to increase the statistical power applied to the data from this trial an advanced statistical test, a repeated measures model, was performed using the FEC results. The details of this test are described in Chapter 2.8.4. When applied to the results of this trial alone, this test provided a statistical indication ($P \leq 0.09$) that there was a real difference in the egg output when the SC and IN groups were compared to the non-vaccinated controls.

Because the control and SC groups were exact repeats of equivalent groups in Trial 1 (Chapter 6), it was considered valid to include the Trial 1 data in the analysis, this constituting a repeated experiment and increasing the statistical power of the analysis. This analysis identified a statistically significant difference in egg output between the SC and IN vaccinated groups and the controls ($P \leq 0.019$). There was no evidence of a difference between the control and IP vaccinated animals and the analysis also indicated that there was no difference between the Trial 1 and Trial 3 results.

8.2.4 Post challenge serum antibody levels

Serum was collected at *post mortem* 11 days after infection and antibody isotype levels were measured by ELISA (Figure 8.2, panels A and B). The titre of all antibody isotypes detected in the serum of AChE vaccinated animals was significantly elevated above those of the control ($P<0.001$). The response was particularly marked with IP delivery being at least 10 fold higher than that with SC or IN delivery (Figure 8.2, panel B). Both IP and SC delivery produced antibody titres that were higher than had been observed during previous experiments (Chapter 6 and 7), but were in a comparable range (Figure 8.2, panels A and B).

IN delivery of AChE resulted in elevated serum titres of each antibody isotype. With the exception of IgA these were higher than those produced by SC immunisation (Figure 8.2, panel A). With all modes of delivery the antibody response was highest in IgG with an increase in IgG1 and IgG2a.



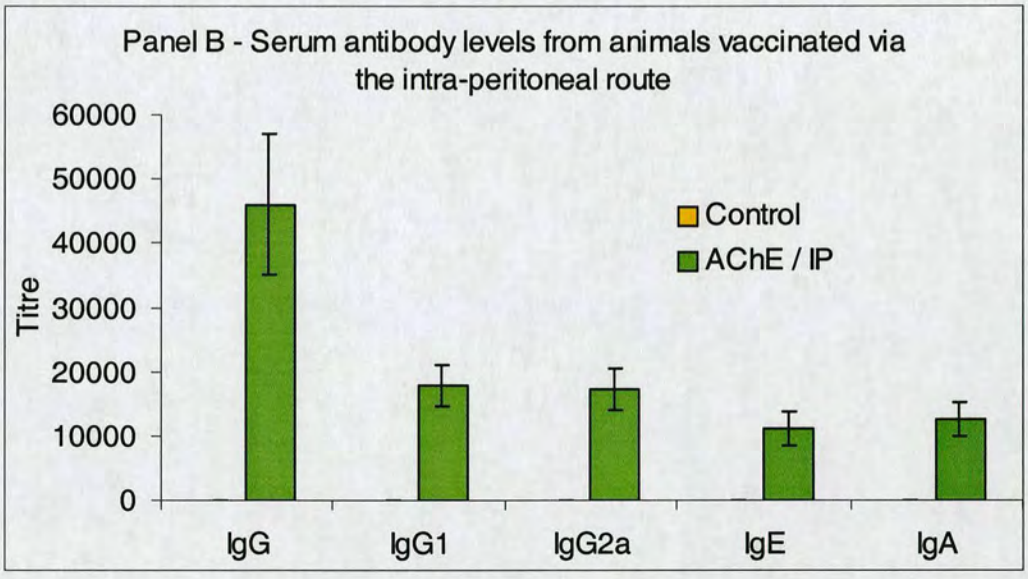


Figure 8.2. The AChE specific serum antibody response of Wistar rats vaccinated with; A.) AChE / SC or AChE / IN and B.) AChE / IP compared with Control (unvaccinated) animals at 11 days following challenge with 25 *Nippostrongylus brasiliensis* L3. The data represent the mean antibody titre of the 7 animals in each vaccination group measured by ELISA (+/- the standard error). Some differences in the levels of specific antibody were observed between the groups.

8.2.5 Mucosal antibody levels

Antibody isotype levels were measured in the mucosa of control and vaccinated animals by ELISA as described in Chapter 2.7.1. Antibody responses were detectable from the mucosa of all of the AChE vaccinated groups, illustrated in Figure 8.3, panel A and B, and these were significantly elevated over the control group titres ($P<0.001$). The highest titres were again produced by IP vaccination, with the titres of mucosal antibody similar to those produced previously (Figure 8.3, panel B, Chapter 7).

Mucosal antibody titres were of a slightly higher level in the IN compared with the SC group for all isotypes except IgG2a (Figure 8.3, panel A). The IgG1 and IgG2a ratio in the IP and SC groups was similar, indicating increases in both subtypes. In contrast to the SC and IP groups and the response observed in the serum, the mucosal IgG response of the IN vaccinated group was dominated by IgG1, approximately 5 times that of the IgG2a titre.

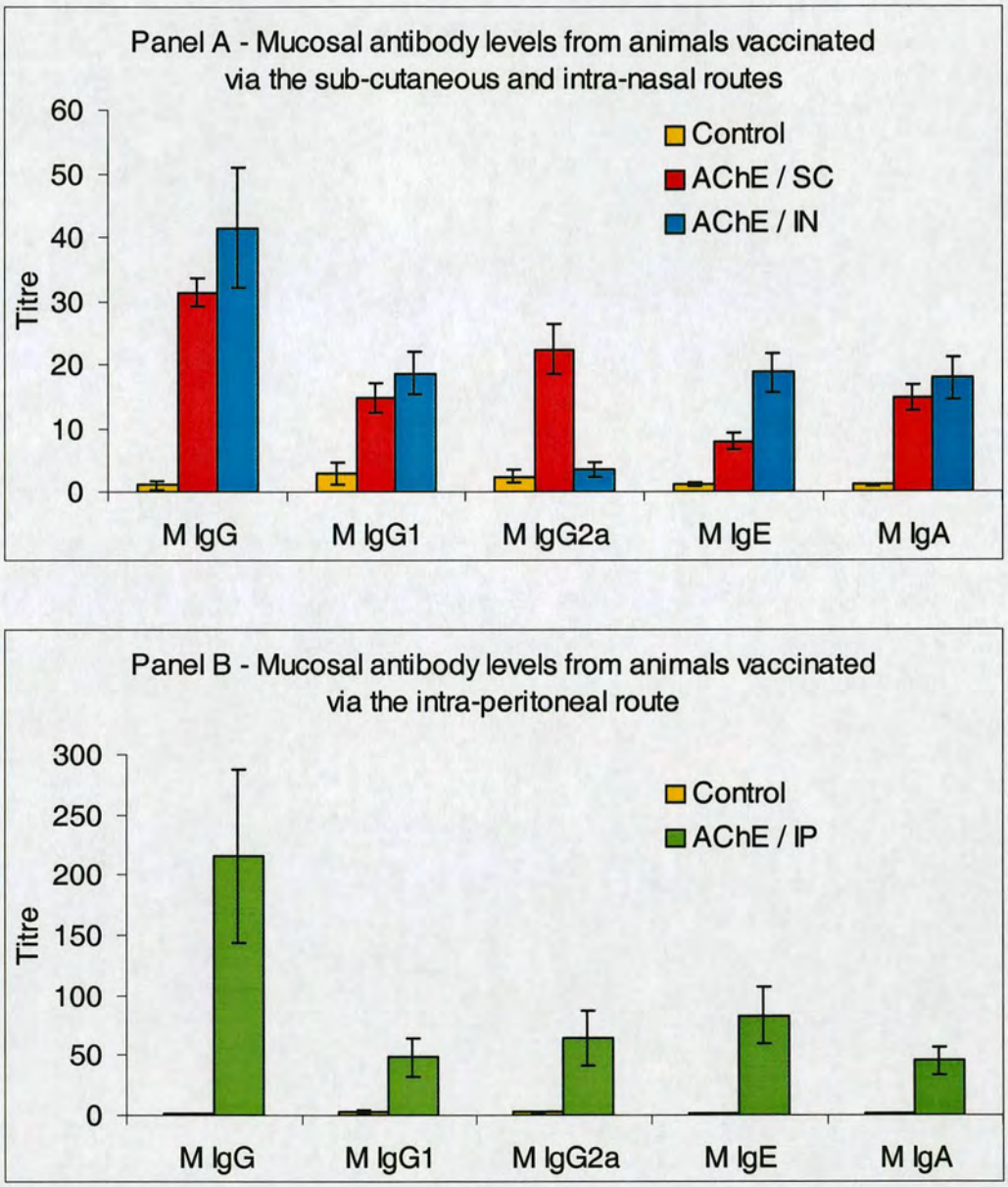


Figure 8.3. The AChE specific mucosal antibody response of Wistar rats vaccinated with; A.) AChE / SC or AChE / IN and B.) AChE / IP compared with Control (unvaccinated) animals at 11 days following challenge with 25 *Nippostrongylus brasiliensis* L3. The data represent the mean antibody titre of the 7 animals in each vaccination group measured by ELISA (+/- the standard error). Some differences in the levels of specific antibody were observed between the groups.

8.2.6 Mucosa and serum Mast Cell Protease levels

Levels of RMCP II were measured from the mucosa and the serum of control and AChE vaccinated animals by ELISA (Figure 8.4).

The amount of RMCP II in the mucosa and in the serum of the unvaccinated control animals was elevated compared with the level previously observed in uninfected animals (Chapter 3) and of a comparable level to those measured from the control animals in the first vaccination experiment (Chapter 6). Serum levels in the SC group and the mucosal levels in all of the vaccinated groups were higher than those observed in the unvaccinated controls however these increases were not statistically significant.

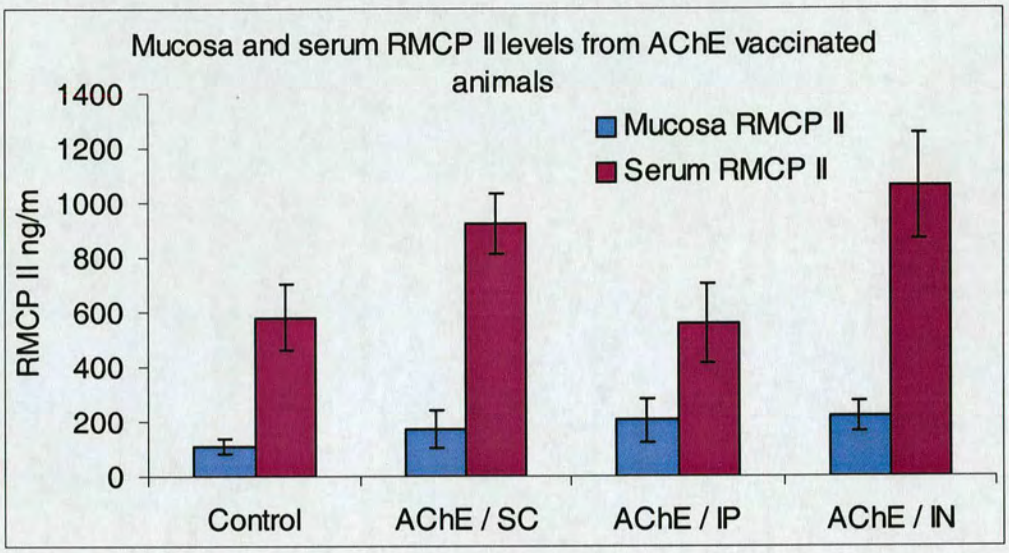


Figure 8.4. The mast cell protease response in the mucosa and serum of Wistar rats vaccinated with AChE and unvaccinated Controls at 11 days following challenge infection with 25 *Nippostrongylus brasiliensis* L3. The data represent the mean protease level of the 7 animals in each vaccination group measured by ELISA (+/- the standard error). No significant differences in RMCP II levels were observed.

8.2.7 Inhibition of acetylcholinesterase activity by antibody

The effect of serum antibody on the activity of AChE was investigated as described previously (Chapter 2.5.5). Native PAGE gels of recombinant AChE and *N. brasiliensis* soluble extract (S1) pre-incubated with sera and stained for esterase activity are displayed in Figure 8.5, panel A - C.

Control serum (Figure 8.5, panel A) showed no effect on the activity of the recombinant AChE as observed previously (Chapter 6 and 7). By contrast, AChE activity was almost completely abolished in the presence of sera from rats immunised with recombinant AChE IN (Figure 8.5, panel B). The effect was similar to those observed when sera from SC and IP immunised animals were tested previously (Chapter 6 and 7).

The effect of serum antibody on the activity of native *N. brasiliensis* enzyme was investigated in the same manner. In this experiment *N. brasiliensis* S1 was separated on the gel either alone or following incubation with serum. Serum from control and AChE vaccinated (SC, IP or IN) was incubated with the S1 for 2 h prior to separation as described previously (Chapter 2.5.5). The Native PAGE gel stained for esterase activity is displayed in Figure 8.5, panel C.

Three bands of activity were visible from the S1 fraction, the lower two of these probably correspond to isoforms of AChE previously identified in *N. brasiliensis* with the upper band representing an endogenous esterase (Edwards *et al.*, 1971). The activity of these bands was unaffected by incubation with the control serum. Incubation with serum from animals immunised SC, IP or IN with AChE however reduced the activity from the lower two bands corresponding to the AChE isotypes termed AChE b and AChE c.

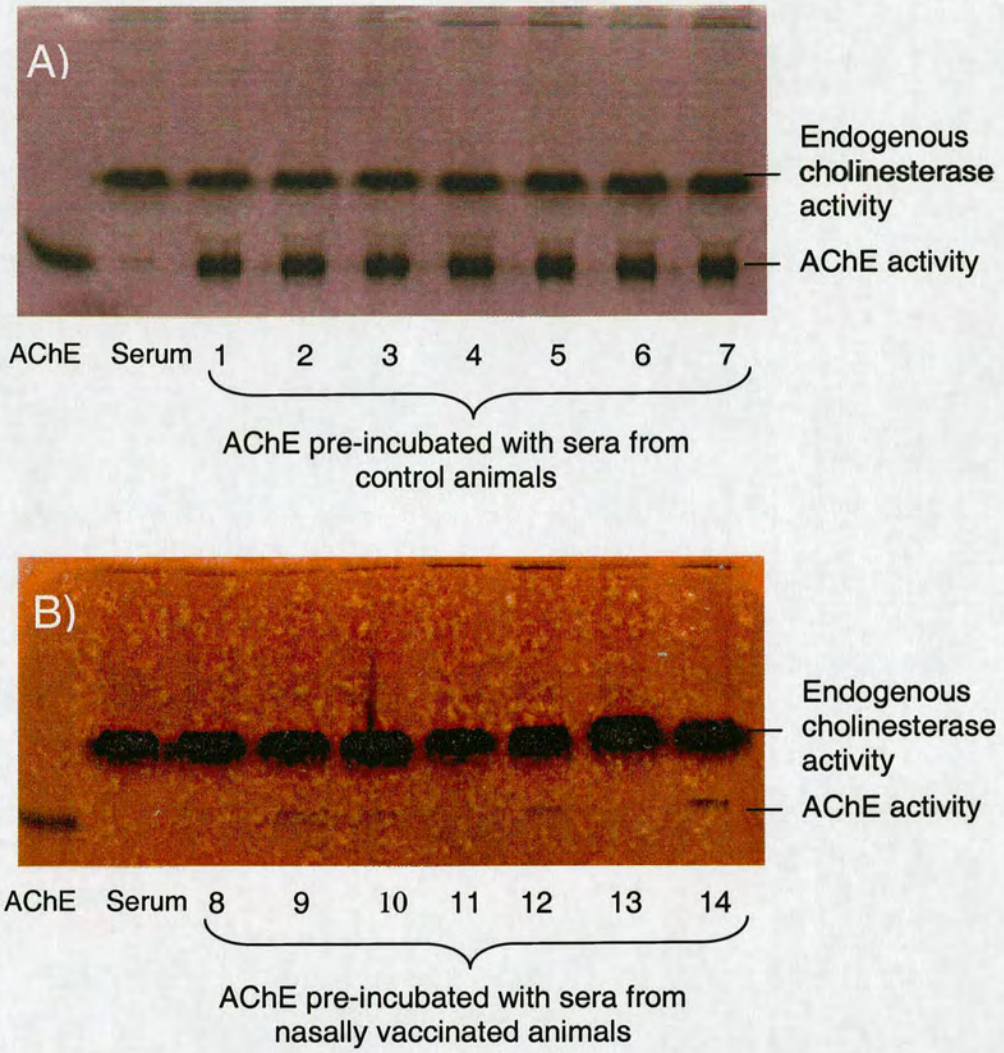


Figure 8.5. The effect of serum antibody on recombinant and native acetylcholinesterase activity. Serum from individual A.) Control (non-vaccinated) and B.) AChE / IN vaccinated Wistar rats was incubated with recombinant AChE, separated on native PAGE gels and stained for esterase activity. The activity of recombinant AChE was reduced by incubation with serum from AChE / IN vaccinated animals (lanes 8 – 14).

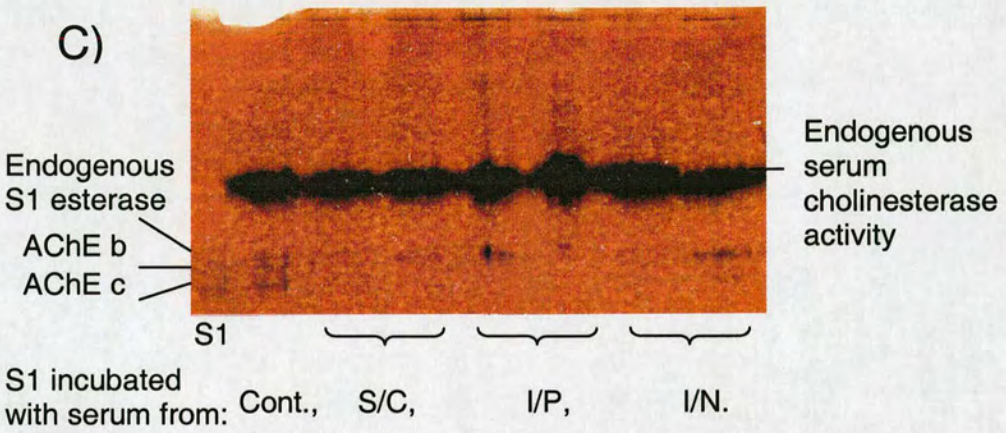


Figure 8.5 (Continued). Panel C.) illustrates the effect of incubation with serum from Control (non-vaccinated) and AChE / SC, AChE / IP or AChE / IN vaccinated Wistar rats on the activity of native acetylcholinesterases (AChE b and c) from a soluble fraction of *Nippostrongylus brasiliensis* extract (S1). The activity of the native AChE was reduced by incubation with serum from AChE vaccinated rats.

8.3 Discussion

The vaccination experiment described in this chapter was established to confirm the protection against *N. brasiliensis* infection in the rat immunised SC with recombinant AChE as described in Chapter 6. Further, this experiment evaluated the immune response and level of protection conferred by the IP and IN routes of antigen delivery. Immunisation via the IP route produces high circulating antibody titres in rats (Harlow and Lane, 1988) and also stimulates a mucosal immune response. IN delivery of antigen in association with CT stimulates Th-2 immune responses (Akhiani, 1997) and has been demonstrated to produce protection against gut dwelling nematodes (Tsuji *et al.*, 2001; McGuire *et al.*, 2002).

In this experiment vaccination with recombinant AChE produced reductions in FEC of between 0 – 38% compared with the unvaccinated control animals. The analysis of these results in combination with results from an earlier trial (Chapter 6) indicated that there was a statistically significant reduction in the FEC of animals vaccinated with AChE via the SC and IN routes. The level of protection produced, as judged by a reduction in FEC, also varied with the different immunisation regimes applied to the three vaccinated groups. These differences in the level of protection were mirrored by differences in the level of specific antibody measured in the serum and the mucosa of the different trial group animals. Antibodies from the serum of the vaccinated groups inhibited the activity of the recombinant enzyme and, importantly, also inhibited the activity of two forms of the native enzyme present in adult worm somatic extracts.

The cumulative FEC from the SC and IN vaccinated groups showed a reduction when compared with the control group (Figure 8.1). This indicated that a degree of protection was conferred by these vaccinations. There was no reduction in the cumulative egg output of the IP vaccinated group. When these data were subjected to an advanced statistical test, the repeated measures test, there was evidence that the reductions in egg output compared to the controls obtained after SC or IN delivery of antigen was likely to be significant. The power of this analysis was enhanced further by combining the data described in this chapter with those from the previous successful vaccination trial (Chapter 6), a valid approach as the groups

represented a repeated experiment under this model. The output of the model showed that a statistically significant reduction in egg output was conferred by immunisation with recombinant AChE using SC or IN delivery.

These results confirm that protection against *N. brasiliensis* infection may be conferred by vaccination with recombinant AChE via the SC route as demonstrated in an experiment described previously in Chapter 6. This is the first report of protective immunity being conferred against nematode infection with AChE alone. In several earlier trials using this antigen equivocal protection results were observed. In a Guinea pig model of *T. colubriformis* infection no significant level of protection was achieved following IP immunisation with an AChE enriched fraction prepared from worm homogenates (Rothwell and Merritt, 1975). Protection of around 50% was achieved in a Guinea pig model of *D. viviparus* infection (McKeand *et al.*, 1995a). This trial used protein extracts enriched for AChE, but also containing other proteins (D.P. Knox, pers. comm.). Protection against *D. viviparus* did not extend to challenged calves when immunised using a recombinant AChE (Matthews *et al.*, 2001). In a mixed gastro-intestinal nematode infection of *T. colubriformis, H. contortus* and *C. oncophora* in sheep, vaccination with partially purified *T. colubriformis* AChE led to a 31% reduction in total worm burden (Griffiths and Pritchard, 1994). Again, the antigen used was not solely AChE.

The results reported in this chapter also indicated that differing degrees of protection were associated with the different vaccination regimes. The least protected group was the IP vaccinated, which showed no overall reduction in egg output. The level of protection conferred by SC immunisation was 23%, compared with 48% in a previous trial (Chapter 6) and the IN vaccinated group gave a reduction in FEC of 38%. Different routes of immunisation have previously been shown to stimulate differing degrees of protection in this system using adult somatic extract as an antigen (Murray *et al.*, 1979). In contrast to the presented results the greatest degree of protection, over 90% as judged by a decrease in the mean number of worms, was afforded by IP immunisation.

Vaccination via the IN route was the most effective mode of immunisation during this trial (Figure 8.1). This confirms previous observations that IN vaccination can provoke protective responses against gut dwelling nematodes (Tsuji *et al.*, 2001,

McGuire *et al.*, 2002). The data presented here, suggest that more effective protection may be achieved with IN delivery than SC and support the hypothesis that locally produced immune responses may be more appropriate for protection against these pathogens. However, IN delivery did also provoke a marked systemic antibody response that may have been active in the protective response demonstrated.

Interestingly, the pattern of faecal egg output differed when each group was compared to the controls. SC immunisation resulted in a delayed peak in egg output compared to the controls (9 days and 7 days respectively) with egg output being lower in the early phase (5 to 8 days) of infection. A similar effect was observed in the IP group but here egg output persisted at a higher level than the controls in the later stages (9 to 11 days) of infection. Following IN delivery, egg output was reduced compared to the controls at all time points throughout infection and showed a similar temporal profile to the controls.

A delay in the onset of peak faecal egg output such as that demonstrated by the SC and IP groups indicates that vaccination with AChE affected the host / parasite interaction, through the immune response or via direct effects on the worm. It might also suggest that the establishment of infection was delayed. As stated in the Chapter 1, AChE has several postulated *in vivo* effects that might aid worm survival. These include acting as a biochemical holdfast by reducing acetylcholine stimulated muscle contraction (Foster *et al.*, 1994) or by modulating immune response mechanisms mediated by acetylcholine such as the induction of rapid mucus secretion from goblet cells (Specian, 1980) and the induction of cellular immune responses (Strom *et al.*, 1974).

Differing serum antibody responses were produced by the different modes of vaccination. The serum IgG response of the IN vaccinated animals was marginally higher than that observed in the SC group and both demonstrated a similar ratio of IgG2a to IgG1. In addition, IgE was higher and IgA lower in the IN compared to the SC group. Systemic antibody responses generated to an IN immunisation, adjuvanted with CT have been previously described (Wu and Russell, 1993, McGuire *et al.*, 2002), but such a response generated by this mode of vaccination may not be the most appropriate for protection against GI nematodes (Emery *et al.*, 1993). IP delivery resulted in the highest titres of each isotype tested, which again were around

ten fold higher than those observed in the other groups (Chapter 7). However, no protection was demonstrated using this route of immunisation.

Measurement of mucosal antibody responses (Figure 8.3) showed that, in contrast to previous results (Chapter 6), all delivery regimes resulted in detectable mucosal antibody. Intriguingly, IN delivery induced a predominant IgG1 response in the IgG isotype and in addition the relative proportion of IgE and IgA was increased in the mucosa. An Th-2 polarised response is considered to be of importance in the expulsion of GI nematodes (Miller, 1984; Finkelman *et al.*, 1997). Increases in specific IgG and IgA in the intestinal mucosa during *N. brasiliensis* infection have been previously documented and correlate with expulsion (Wedrychowicz *et al.*, 1983). Increases in intestinal IgA following IN immunisation are also associated with protection against *A. suum* and *T. spiralis* (Tsuji *et al.*, 2001; McGuire *et al.*, 2002). It is interesting to speculate that the observed alterations in the antibody isotype ratio at the site of parasite infection contributed to the slightly higher level of protection observed in the IN immunised group during this trial.

The relative proportions of antibody isotypes observed when serum and mucosal responses were compared, in particular the observed shift in the IN group to an IgG1 dominated response, suggest that some of the mucosal antibody was produced locally as a result of this mode of antigen delivery. The relative proportions of antibody in the serum and mucosa were the same for both the SC and IP groups. This indicates that the antibody present in the mucosa of these groups was likely to be derived from systemic leakage into the mucosa as a result of worm damage or the resultant immune response (McDermott *et al.*, 2003). This hypothesis is further supported by the mucosal antibody of the SC group correlating with the increased serum antibody response in this trial.

The apparent differences in the class and / or origin of the mucosal antibody response in the IN group might suggest that there are important differences in the ability of this antibody to carry out it's protective function. These might include increased avidity such as has been demonstrated in the vaccination against *F. hepatica* using Cathepsin-L protease (Mulcahy *et al.*, 1998). If so then this would indicate that the amount (relative titre) of antibody is less important than the quality of this antibody. This might also explain why, though the IP vaccinated group clearly

shows a high response to vaccination and changes the profile of egg output, it does not confer an overall protective effect i.e. in this case the response is not appropriate. It may also be that with the IN vaccinated group that the antibody *per se* is not the effector of protection. The differences in antibody in the mucosa might merely reflect that there is a difference in the response of this group and that some other component of this different response confers increased protection.

Serum antibody from IN vaccinated animals inhibited the activity of AChE (Figure 8.5). Similar inhibition was observed by antibodies in the sera of guinea pigs immunised against *D. viviparus* (McKeand *et al.*, 1995b) and has been demonstrated by the sera of sheep infected with a variety of nematode species (Rothwell *et al.*, 1976). There was a suggestion that serum from IN vaccinated rats inhibited AChE activity to a greater extent than serum from those vaccinated by SC (Chapter 6) but less so than serum from IP vaccinated animals (Chapter 7). These differences probably reflected differences in the serum antibody titre between the samples. However, an experiment intended to confirm this did not resolve the cause due to the confounding effects of endogenous serum esterase activity, which masked the effects of the titration. The observed differences in antibody inhibition of the AChE activity might again be due to class differences in the antibody response stimulated by the different vaccination regimes. Factors such as increased avidity of the IN serum antibody might produce the observed differences in inhibitory effect. Measurement of the avidity of antibody can be performed by thiocyanate elution as described by Mulcahy *et al.* (1998).

Importantly, the serum antibody from all three modes of vaccination inhibited the activity observed from two forms of AChE present in a somatic extract of adult *N. brasiliensis* worms. This indicates that the antibody produced by these immunisations inhibits the native AChE enzyme as well as the recombinant form. During infection native AChE enzyme illicits an IgE and IgG1 antibody response in the host (Nakazawa *et al.*, 1995). This response has been shown to affect the profile of enzyme isoform expressed by the worm with associated effects on the viability of the worms (Jones and Ogilvie, 1972; Sanderson *et al.*, 1972). The finding that antibody from vaccinated animals has an effect on native enzyme activity therefore

supports a role for the antibody response produced by vaccination with AChE in the protective response observed.

No significant differences in the level of RMCP II between the control and vaccinated groups were observed during this experiment. This contrasts with the previous successful vaccination trial (Chapter 6) in which serum levels of the protease in vaccinated animals were reduced compared to the controls. As a higher level of protection was observed in the previous trial as judged by egg output, the reduced serum RMCP II level in that trial might have been indicative of a reduced worm burden and therefore reduced stimulation of mast cell response.

The results obtained in this trial confirmed that a reduction in egg output and therefore a protective effect was conferred by immunisation with recombinant AChE. The results further indicated that different modes of immunisation might confer varying levels of protection and that these might be associated with changes in the serum and mucosal antibody level. Further, the serum antibody was shown to inhibit the activity of the recombinant enzyme and subtypes of AChE in the somatic extract of *N. brasiliensis*. This work therefore confirms that AChE might be a useful antigen in the production of a recombinant vaccine against GI nematode infection. The mode of application of anti-nematode vaccines was also shown to be important in the candidate antigens ability to generate protective immune responses. Nasal vaccination produced the highest degree of protection and appeared to stimulate a local immune response. These findings support further investigation of this route of immunisation for delivery of anti-nematode vaccines.

Chapter 9 – General Discussion

GI nematode infections in farm animals are a cause of substantial production and economic loss both in the UK and world-wide. Control is currently undertaken using prophylactic dosing regimes using three classes of anthelmintic drugs. Spreading parasite resistance to all three classes of these anthelmintic drugs along with consumer concerns over chemical residues in food has driven interest in the production of vaccines as an alternative means of controlling these infections. The use of recombinant proteins as a potential source of antigen might yield a relatively cheap means of production of successful vaccine candidates (Knox, 2000; Knox *et al.*, 2001).

In the UK two species of nematode are of particular importance in sheep, the abomasal dwelling species *T. circumcincta* and the small intestinal species *T. vitrinus* (Reid and Armour, 1975; Bartley *et al.*, 2003). The related rat intestinal nematode *N. brasiliensis* shares many similarities with species of the genus *Trichostrongylus*, including the niche it occupies in its host and, importantly, in the functional antigens that it excretes or secretes. Several of these antigens, including the enzymes AChE and SOD, have been identified as potential vaccine candidates against different species of GI nematode and have been tested in vaccination trials in sheep (e.g. Griffiths and Pritchard, 1994; Liddell and Knox, 1998).

Vaccination trials in sheep are expensive and this therefore limits the parameters, such as different antigens or modes of delivery that can be tested. The aim of this project was therefore to evaluate *N. brasiliensis* infection of the rat as a suitable model to test recombinant protein vaccines, primarily against *Trichostrongylus sp*. This system might offer the advantages of being cheap and easy to manipulate as well as allowing extended trials, such as the investigation of different modes of antigen delivery. Immunological markers in the rat are also well defined and easily measured.

Experimental challenges with *N. brasiliensis,* described in Chapter 3, initially confirmed that the proposed model infection behaved as had previously been described (Ogilvie and Jones, 1971). Parasitological measures, the faecal egg output and the number of adult worms established in the intestine, peaked at 7 days after

which both declined as expulsion progressed. The pattern was the same irrespective of the level of infection employed. Egg output could be accurately determined and showed low variability at low infection doses. The FEC was subsequently chosen as the primary measure of vaccination challenge infections. This measure can also be performed without the need to sacrifice animals at each time-point, an important practical consideration given the cost of animal purchase and upkeep and reduction in animal numbers for welfare considerations.

Challenge experiments with varying numbers of L3 larvae in several regimes of low-level infection allowed the most appropriate dose of larvae with which to challenge vaccinated animals to be decided (Chapter 3). Doses of around 2,000 L3 have previously been used in laboratory infections (Ogilvie and Jones, 1971) but such a large challenge might overwhelm any protective responses conferred by immunisation. An infection using 25 L3 larvae, yielding a consistent egg output that lasted for over 12 days was subsequently chosen as an appropriate challenge dose.

Trickle infection regimes with small numbers of larvae have previously been shown to lead to a cumulative and prolonged infection (Jenkins and Phillipson, 1971; 1972). This situation has clear similarities to infection establishment and maintenance in the ruminant where animals are continuously exposed to infection from pasture. In the present study, despite using several variations of this regime, it proved to be impossible to provoke a cumulative, chronic infection.

It has been suggested that the accumulating worm burden achieved in these previous studies may have been an artefact caused by the young age of the challenged animals (Miller, 1984). Prior to 11 weeks of age, rat pups do not expel secondary infections of *N. brasiliensis* as efficiently as older animals (Jenkins, 1974). In these experiments, however, all animals were pre-immunised with a *N. brasiliensis* infection of 1,000 L3 larvae prior to the challenge infections described (Jenkins, 1974). In the trickle challenge experiments of (Jenkins and Phillipson, 1971; 1972) there was no immunising infection and trickle challenges produced cumulative worm burdens and egg counts in naïve animals.

The data presented in this study suggest that it may be possible to provoke a chronic *N. brasiliensis* infection, as a low egg count was maintained through the course of an infection regime in which animals were infected with 5 L3, three times a

week. Sterile immunity was not provoked, however a cumulative infection such as that previously described (Jenkins and Phillipson, 1971; 1972) was not achieved. The reason for this appeared to lie in the extremely critical level of infection needed to produce a cumulative worm burden without stimulating an expulsive immune response (Chapter 3).

Apart from measuring the effect of vaccination on infection outcome using parasitological measures, there was a need to develop assays to monitor the humoral and cellular immune responses in the challenge experiments. If protection were noted, it might correlate to a particular immune parameter. This information might suggest a mechanism of protection and indicate an antigen delivery method for future experiments, further enhancing the protective response.

Methods to measure some appropriate immunological markers, selected on the basis of work published previously, were developed (Chapters 3 and 5). These included several subtypes of parasite-specific antibody and a mast cell protease (RMCP II). Analyses were conducted on samples of serum and small intestinal mucosa, the former to provide an indication of the systemic, the latter the local, small intestine mucosa immune response. In Chapter 3, some of these assays were used to analyse the responses of rats to challenge infection. These experiments demonstrated increases in serum antibody responses and RMCP II levels during the course of infection similar to those described previously (Ogilvie and Jones, 1971; Miller, 1984). Importantly, a rise in IgG1 titre correlated closely with the time of expulsion of the worms. This isotype has previously been linked with protection (Jones *et al.*, 1970) and is indicative of a Type 2 immune response (Chapter 3).

Increases in the level of RMCP II in the serum and in the intestinal mucosa also correlated with expulsion of the worms. RMCP II is released by activated mast cells and may be involved in the increase in epithelial permeability of the intestine observed (McDermott *et al.*, 2003). It has been suggested that this facilitates the expulsion of GI nematode infection by allowing the transport of serum components (such as antibody) and fluid into the intestinal mucosa and degrading the environment of the worm. RMCP II release is strongly stimulated during *N. brasiliensis* infection and is indicative of a Type 2 response (Miller *et al.*, 1983b); (Huntley *et al.*, 1993).

Cytokine production can provide information on the Th balance of an immune response. Th-2 cytokines are considered to be produced during and influence the immune response and outcome of nematode infection (Urban *et al.*, 1992). Th-2 cytokine production has previously been observed during *N. brasiliensis* infection (Matsuda *et al.*, 1995; 1999). Despite extensive effort, a RT PCR method for detection of cytokines from infected rat tissues (Matsuda *et al.*, 1995) did not prove be a consistent or repeatable method for measurement of this immune marker set during the present study (Chapter 5). An alternative approach would be to measure the levels of cytokine protein in the mucosal extracts using commercially available anti-cytokine antibodies and ELISA based assays.

Reactive oxygen intermediates (ROI) such as O_2^- and OH^- are thought to play a role in the host immune response, and their production by a number of cells, including macrophages and eosinophils, has been detected (reviewed; Rothwell, 1989). ROI have been implicated in worm expulsion through mechanisms that may either alter the intestinal environment by damaging intestinal cells and rendering it unsuitable for parasite maintenance or by damaging the worms directly. Damage to worms could be effected by ROI reducing levels of protective enzymes and inhibiting lipoprotein synthesis (Smith and Bryant, 1989b) or causing DNA damage and lipid peroxidation in membranes (Nussler and Thomson, 1992).

The susceptibility of different nematode life cycle stages to *in vitro* killing by granulocytes and oxidant mediated killing appears to be dependent on the levels of SOD and other antioxidant enzymes in the ES (Lightowlers and Rickard, 1988). Comparison of rodent infections with *N. dubius* (formerly *H. polygyrus*) and *N. brasiliensis* has implied a role for antioxidant enzymes including SOD in the longevity of infections (Smith and Bryant, 1986). Higher levels of antioxidant enzymes were detected in *N. dubius* compared to *N. brasiliensis*, indicating a possible reason for the persistence of *N. dubius* infections while *N. brasiliensis* infections were expelled (Smith and Bryant, 1986; 1989a; 1989b). In *S. mansoni* infections parasite antioxidant levels increase as the parasite matures and adults appear less susceptible to oxidative damage (Nare *et al.*, 1990).

Generation of ROI by activated phagocytes has been demonstrated to occur in *N. brasiliensis* infections and is observed earlier and at greater levels during heavy

compared with lighter infections (Smith and Bryant, 1989a). In *N. brasiliensis* infections SOD levels have been demonstrated to decrease while infection progresses and rising oxidant levels were detected in the host intestine, these changes being implicated in worm expulsion (Batra *et al.*, 1993). The SOD isoform profile also varies with age and with host immune status as activity and isoform polymorphism increased in primed compared with naïve host (Knox and Jones, 1992).

The evidence therefore appears to suggest that antioxidant enzyme levels play a role in preventing worm expulsion in a number of species. In addition lambs immunised with enzymically active recombinant SOD protein from *H. contortus* were partially protected against challenge infection (Liddell and Knox, 1998). For these reasons, SOD was selected as one of the main antigens of interest for use in the vaccination studies described here.

The cloning and expression of a putative SOD gene from *N. brasiliensis* cDNA for use a recombinant vaccine was described in Chapter 4. Homology analysis showed that the gene was a cytoplasmic form of the enzyme (SODc), through its close homology to other nematodes SODc and its lack of a 5' transmembrane leader sequence, carried by extra-cellular isoforms (Liddell and Knox, 1998; Henkle-Duhrsen *et al.*, 1997). The expressed bacterial recombinant was soluble and enzymically active, both of which features might be important in its effectiveness as a vaccine candidate. The predicted protein sequence showed high homology with a putative extra-cellular isoform for which a partial sequence was obtained. It was therefore considered suitable to proceed with vaccinations using this cytoplasmic form of the enzyme, in the absence of a cloned extra-cellular isoform. Antibodies rasied against the *H. contortus* SODc cross-reacted with the extra-cellular form from the same parasite (Liddell and Knox, 1998). It was therefore considered likely that the two *N. brasiliensis* forms would be antigenically similar, due to their high homology, with the result that a vaccine induced immune response would be effective against both forms.

Acetylcholine acts as a neurotransmitter in the central and peripheral nervous systems in humans and animals. Recent experiments have demonstrated a widespread expression of the cholinergic system in non-neuronal cells in humans (Wessler *et al.*, 1998). Choline acetyltransferase, the synthesising enzyme,

acetylcholine, and acetylcholine receptors are expressed in a variety of cells and non-neuronal acetylcholine appears to be involved in the regulation of many important cellular functions. Importantly it also has barrier and immune functions.

Esterases hydrolyse uncharged substrates such as lipids or acetylcholine and are located throughout the tissues. Esterases have been detected in nematodes including *T. colubriformis* (Rothwell *et al.*, 1976), *N. brasiliensis* (Edwards *et al.*, 1971) and *D. viviparus* (McKeand, 1992). The cholinesterases hydrolyse esters of choline and carboxylic acids and are separated into two groups, the true cholinesterases and pseudo-cholinesterases. AChE is a true cholinesterase and hydrolyses acetylcholine as a substrate. AChE in nematodes has a 'house keeping' role in modulating acetylcholine levels at neuronal and neuromuscular junctions. Secreted AChE may have a number of further possible functions. These include modulating mucus secretion, inhibiting some anti-parasite immune responses and functioning as a 'biochemical holdfast' by inhibiting local gut peristalsis (reviewed Pritchard, 1993).

AChE release during *in vitro* maintenance has been recorded from a number of GI nematodes including *N. brasiliensis, T. circumcincta, H. contortus,* and *T. colubriformis* (Ogilvie *et al.*, 1973). Evidence of AChE release by *D. viviparus* and *T. vitrinus* has also been provided (McKeand *et al.*, 1994b; Jones and Knox, 1990; MacLennan, 1995). Immunolocalisation studies have demonstrated that AChE in *N. brasiliensis* is concentrated in the sub-ventral glands, which insinuates active secretion of the enzyme (Nakazawa *et al.*, 1995). *In vivo* release of AChE is implied also by the observation of parasite AChE-specific antibodies in immune animals (Jones and Ogilvie, 1972; Rothwell *et al.*, 1976; McKeand *et al.*, 1994b).

McKeand *et al.* (1994a) noted that isoenzymes of AChE in *D. viviparus* showed antigenic differences and suggested that this may allow parasite AChE to remain active in the face of antigenically specific immune responses which could effect parasite survival. Differential expression of secreted AChE isoforms has also been observed during *N. brasiliensis* infection (Edwards *et al.*, 1971; Blackburn and Selkirk, 1992). The isoform profile was observed to alter during the course of an infection and this correlated with the expulsion of the worms and the host immune status (Edwards *et al.*, 1971; Sanderson *et al.*, 1972). Residual "adapted" worms that

are able to overcome the immune response had an increased and altered AChE expression profile from normal worms

Elevated levels of non-specific esterases, with a relatively broad substrate specificity, have been detected in *H. contortus, T. circumcincta* and *T. colubriformis* strains resistant to benzimidazoles (BZ) compared to parasites which were susceptible to the drug (Sutherland and Lee, 1993). Why esterase levels are increased is unknown, but it has been suggested that the enzyme is involved in the detoxification of BZ or alternatively that BZ binding to tubulin may interfere with microtubule formation which may impair enzyme secretion (Lacey, 1988). Resistance to levamisole-morantel anthelmintics has also been associated with raised levels of AChE in the parasite (Sutherland and Lee, 1993) further indicating the potential importance of AChE for parasite survival.

These observations give an indication that the secretion of AChE is important in the *in vivo* maintenance of GI nematodes. As a result, AChE has been proposed as a potential immunogen, for use in sero-diagnosis or vaccination (Jones and Knox, 1990; Knox, 1994). For these reasons, AChE was selected as a second antigen on which the present studies would be based

The enzyme used in the work described here was a kind gift from Prof. Murray Selkirk, Imperial College, London. High level expression was obtained via secretion from *Pichia pastoris* (Hussein *et al.*, 1999). The purified enzyme (isoform B) behaved as a monomeric hydrophilic species. The recombinant enzyme efficiently hydrolysed acetylthiocholine but showed minimal activity against the alternative substrate butyrylthiocholine. It also displayed excess substrate inhibition with acetylthiocholine at concentrations over 2.5 mM and was highly sensitive to both active site and "peripheral" site inhibitors (Hussein *et al.*, 1999).

Vaccination trials using the recombinant SOD and the recombinant AChE are described in Chapters 6, 7 and 8. In repeated trials (Chapter 6 and 8) vaccination with AChE reduced cumulative egg counts by 48% and 23% respectively. Although individually results were not significantly lower than the control, statistical analysis of combined egg count data from SC and IN immunisations indicated that a significantly reduced egg output was conferred by these treatments. These reductions in faecal egg output indicated a protective effect and confirmed the utility of AChE

as a vaccine. This follows previous successful trials with AChE-enriched fractions against *D. viviparus* in Guinea pigs (McKeand *et al.*, 1995a) and mixed infections containing *T. colubriformis* in sheep (Griffiths and Pritchard, 1994). However, importantly this is the first demonstrable protection conferred solely by a recombinant AChE antigen, suggesting that a recombinant AChE may be efficacious against ruminant infections.

The single trial with IN immunisation of the AChE reduced egg output by 38%, while the observed data indicated that no significant reduction in the egg output was conferred by IP immunisation (Chapter 8). These regimes tested the hypothesis that targeting immunisation to mucosal surfaces might promote more appropriate immune responses to GI nematodes (Emery *et al.*, 1993). The data confirm that IN delivery of protein vaccines can elicit protection in rodent nematode infections (Tsuji *et al.*, 2001; McGuire *et al.*, 2002). The level of protection was higher than the SC group produced in this experiment (38% compared to 23%), therefore IN immunisation might offer improved levels of protection when compared directly with SC immunisation (Chapter 8).

SC and IN immunisation induced elevated titres of specific antibodies in the serum (Chapter 6, 7 and 8). Specific antibody in the mucosa of SC vaccinated animals was also detected in the final trial (Chapter 8). In this latter instance it was possible that the antibody was a result of crossover from the serum and not local antibody production because the relative ratio of the isotypes was similar in both compartments with IgG2a predominating (Chapter 8, Figure 8.3 and 8.4). By contrast, while IgG2a predominated in the systemic response to IN delivery, the mucosal response showed predominant IgG1 and elevations in IgE and IgA, indicative of a local mucosal Th-2 type antibody response. Because the protection observed in the IN vaccinates was higher that that in the SC, it is possible that the IgG1 isotype is the principal effector antibody in the context of a pronounced Th-2 background.

IP immunisation induced a relatively very large rise in specific antibody isotypes in the serum that was mirrored by an increased mucosal antibody level (Chapter 7 and 8). Once again it was possible that the mucosal antibody was a result of crossover from the serum. The lack of protection observed suggested that this

extremely strong serum antibody response to immunisation was not appropriate to elicit protection.

As noted above, in contrast to the other modes of immunisation the antibody response in the mucosa of IN immunised animals was differentiated to an IgG1 response, with an increased IgE and IgA component. This suggested a local antibody response with a bias towards Th-2 response. Such a response has been previously demonstrated using this adjuvant (Cholera toxin) via this route of immunisation and might be more appropriate in stimulating responses against GI nematodes (Tsuji *et al.*, 2001; McGuire *et al.*, 2002). It might be that the elevated level of protection compared with IP and SC vaccination (38% compared with 0 and 23% respectively) was conferred by these local changes in antibody profile or that these were indicative of an improved protective response. The IN group did also however express a serum response that was slightly elevated compared with the SC.

The involvement of a local mucosal immune response in protection could be confirmed by defining local antibody production from antibody secreting cells harvested from the draining (mesenteric) lymph nodes (Bowles *et al.*, 1995). In addition, cytokine expression in these nodes could be monitored using a RT PCR assay, as attempted in Chapter 5. Indeed, using dissected out lymph nodes would provide a richer source of cytokine mRNA than the whole mesentery tested in Chapter 5 and may overcome the difficulties of specificity and sensitivity indicated by that work.

In this study, measurement of the levels of RMCP II in the sera and intestinal mucosa of rats in each of the vaccination trials was used as a marker of cell-mediated immunity. Elevations in the levels of this enzyme have previously been shown to coincide with worm rejection (Miller, 1984; Balic *et al.*, 2000). In the present study, RMCP II proved to be an inconsistent marker during the vaccination trials. For example, although serum levels were depressed in the AChE immunised group in the first trial (Chapter 6, Figure 6.5) compared to control rats and indicated a significant alteration in the activation of mast cells, in a later trial serum RMCP II levels were elevated (Chapter 8, Figure 8.4). Similar contrasting results arose from the measurement of mucosal levels of the enzyme. It is likely that these data fall within the normal range of variation associated with RMCP II levels in the rat, a suggestion

supported by the range of levels observed in the control groups in the three vaccination experiments conducted. The titres of RMCP II observed in each of the trials was much lower than those observed at the 11 DPI peak noted in Chapter 3, although this is probably explained by the much lower challenge infection.

From the observed changes in antibody isotype levels and the lack of an observed cellular response, it is possible to draw the tentative conclusion that the effectors of protection observed was an antibody response to the parasite enzyme inhibiting its action. That antibody against AChE is involved in protective immunity has been indicated previously in *N. brasiliensis* and other parasites known to secrete AChE (Jones and Ogilvie, 1972; Rothwell *et al.*, 1976; McKeand *et al.*, 1994b).

Evidence that this was a possibility was sought by employing a gel based enzyme activity assay. With the gels run under non-reducing, non-dissociating (Native) conditions, enzyme-inhibitor (antibody) complexes should not be disrupted with the result that migration of the enzyme in the gel would be much slower than the native enzyme, or the activity may impaired. In this study, enzyme migration was retarded by pre-incubation with sera from animals vaccinated with recombinant AChE (Figure 6.6). An additional slow-migrating zone of activity was apparent at the top of these gels compared to controls, indicating that the enzyme was complexed to a factor in the serum, an interaction that did not inhibit activity. Activity was inhibited by pre-incubation with sera from animals vaccinated with recombinant AChE via the IP and IN routes (Figure 7.5 and Figure 8.5), possibly due to the higher antibody titre of these sera or some qualitative difference in the antibody (Chapter 7 and 8). Importantly, this inhibition was also apparent when native AChE in parasite extracts was incubated with vaccinate sera (Figure 8.5, panel C).

Whether or not the factor responsible for this retarded migration and inhibition was antibody could be confirmed by purifying antibody from the sera using Protein G. Similar inhibition of *D. viviparus* AChE by sera from vaccinated Guinea pigs was attributed to IgG by such a method (McKeand, 1992).

Repeated trials indicated that the recombinant SOD did not induce a strong immune response as judged by the antibody response to systemic (SC) immunisation. Further to this, the immunisation produced no reduction in egg output and the cellular response (as judged by RMCP II level in serum and mucosa) did not differ

significantly from the control. One possible reason for this lack of response is genetic restriction of antigen recognition in the strain of rat being used. This hypothesis being supported by an earlier finding that different, hyper-immune animals varied in their recognition of the expressed SOD enzyme (Chapter 4).

That genetic restriction might be responsible for the lack of response to immunisation with the recombinant SOD was examined by immunisation and challenge of a second strain of rat (Sprague-Dawely). Again, the IgG responses were not significantly increased compared to the control or responses in the original rat strain (Wistar). Therefore, it is likely that the SOD protein is simply weakly immunogenic in its present form. Methods by which the immunogenicity of this protein could be improved were described in Chapter 7.3.

The results obtained in this series of experiments suggest that the *N. brasiliensis* / rat infection system might be a useful model of vaccination against intestinal nematodes. The antibody response comprised of increases in specific IgG, IgE and IgA, with increases in IgG1 and IgG2a correlating with expulsion (Chapter 3). Mast cell activation with release of protease also correlated with expulsion indicating a cellular component to the response (Miller *et al.*, 1983b). Antibody and mast cell responses in the rat during this infection were therefore confirmed as being similar to those produced during ruminant nematode infections (reviewed; Miller, 1996; Balic *et al.*, 2000).

Immunisation with the recombinant AChE produced an immune response that conferred a protective effect (Chapter 6 and 8). As discussed previously AChE may have several *in vivo* functions in the maintenance of the parasite within its host and is excreted or secreted in relatively large amounts by both *N. brasiliensis* and *Trichostrongylus sp.*, an indicator of its functional importance (Ogilvie *et al.*, 1973). The observed protection when either host is immunised with this protein strongly suggests that the enzyme is of importance in the maintenance of infections with both species and is suitable as a vaccine candidate. These observed similarities support the utility of the *N. brasiliensis* / rat model system outlined in this thesis for the investigation of vaccines against *Trichostrongylus sp.*

The lack of response demonstrated following vaccination with SOD is in contrast to a previous trial using *H. contortus* SOD (Liddell and Knox, 1998) and the

suggested role of SOD in the maintenance of this infection (Henkle-Duhrsen and Kampkotter, 2001; Smith and Bryant, 1986). These data indicate that isolation of a recombinant secreted isoform or improvement of the immunogenicity of the protein may need to be carried out to truly assess the utility of this vaccine candidate.

It is the similarity between the two intestinal species that is of primary consideration in assessing the potential of *N. brasiliensis* as a model for *Trichostrongylus sp.* Ultimately it is by comparison with vaccination trials against *Trichostrongylus* in sheep that this system must be judged as a useful vaccination model of intestinal nematodiasis. The level of protection conferred by immunisation with AChE during this trial and in the previous studies using this antigen may be comparable (approximately 30-50%; Griffiths and Pritchard, 1994; McKeand *et al.*, 1995a). However in these previous trials protection was measured as a reduction in worm burden rather than egg output. A further extended study to measure any reduction in worm burden conferred by vaccination with *N. brasiliensis* AChE would therefore be required to allow a direct comparison of these experiments. This could be achieved by expanding the current trial design to include killing rats at 7 days post infection to harvest and count the worms present in the small intestine.

Immunising animals via different routes may influence the expressed immune response, with consequent effects on the protection achieved (Murray *et al.*, 1979; Tsuji *et al.*, 2001). As mucosal responses may be more important in protection than a systemic response, rejection of potentially useful antigens based on lack of protection conferred when tested using a systemic immunisation regime is a danger. As equivocal results using the same antigens with different adjuvant may be obtained, this system might be of use in screening the most appropriate adjuvant (Jacobs *et al.*, 1999; Vervelde *et al.*, 2003). Investigation of different modes of immunisation indicated that different degrees of protection are conferred using different routes of immunisation and different adjuvants in the *N. brasiliensis* system (Chapter 7 and 8). Once again, this supports the utility of this model system in the investigation of different modes of delivery and adjuvant formulation for ruminant vaccine candidates.

Further uses of this system might include the investigation of the effects of parturition on the outcome of vaccination (Houdijk *et al.*, 2003). The outcome of

vaccination in neonatal rats, which are unable to expel infections (Love and Ogilvie, 1974) in a similar manner to young lambs (Gibson and Parfitt, 1972), could also be investigated. As discussed in Chapter 1 these are epidemiologically important phenomena in ruminant infections.

Screening other candidate antigens with homologues in *Nippostrongylus* could also be carried out. These include the *Ancylostoma* secreted protein (ASP) homologues, which are ES proteins of interest in vaccination studies against the human hookworm *Ancylostoma duodenale* (Hawdon *et al.*, 1996), and the ruminant GI nematode *H. contortus* (Schallig *et al.*, 1997). Further, as a rodent hookworm, the *N. brasiliensis* infection model might also have relevance to studies of hookworm vaccines, due to the similarities in life cycle and gene expression. ASP homologues are present in the *N. brasiliensis* EST data set (www.nema.cap.ed.ac.uk.org.nematodeESTs.nembase.html), but the outcome of trials with these or other candidates might depend on the relevance of the antigen in the life cycle of the worm.

While these findings may be useful there are a number of problems that exist with the model and that would need to be addressed to allow full assessment of its potential. The primary concern identified by these experiments was that the variation in egg counts from vaccinated groups of animals at each timepoint was so large that it was difficult to identify meaningful statistical differences between treatments. While this problem was overcome by combining trial results in analysis, the effect was primarily due to the large variability of the data, and the low number of samples that could be collected. Increasing the number of samples from each group would be necessary to address this problem (Jill Sales, BioSS, pers. comm.). A sample size of seventeen per group has been estimated to be necessary to resolve statistically significant differences between two groups of animals based on the data from these trials. This sample number decreases as more groups are included in each trial, with fifty animals in total estimated for four trial groups (Jill Sales, BioSS, pers. comm.).

Although during these trials seven animals were included in each group, restrictions on housing due to welfare and Home Office licensing restrictions meant that they were caged in pairs or threes. This limited the sample number to three samples per group, thereby limiting the statistical power that could be applied to the

data. One way in which this problem could be removed is by housing animals individually during the course of challenge infection or, at least, during faecal sampling periods. Individual sampling would allow an increased number of samples per group, compared with the three possible during these trials. Correlation of high and low egg output to the response of immune markers in individuals could also be detected, allowing more insight into the possible mechanisms of protection. With the trials performed in this study as a pilot experiment, this housing scheme could be justified in future experiments.

The restriction on using sawdust floors instead of wire mesh also may have limited the quality of the faecal samples due to drying and meant more regular and time consuming sampling techniques had to be used. The use of wire mesh floors over damp towelling would allow less frequent and higher quality samples to be collected making the experiments easier to perform and possibly increasing the quality of the data (reducing variability). Housing animals on wire mesh floors in this manner would also allow a larger sample to be collected per animal, as coprophagy would be eliminated. This would also eliminate any possible auto-reinfection caused by worm eggs in the faeces. Once again, with the trials performed in this study as a pilot experiment, housing on mesh floors, at least during the sampling period, could justify these changes in future experiments.

Other changes to the sampling regime might also be useful. The FEC data presented here indicated that a major difference between vaccinates and controls occurred at 7 DPI (Chapter 6 and Chapter 8). In future trials, antigens could be screened initially for protective efficacy by measuring faecal egg output in faeces harvested directly from the colon of individual animals in each group. This would increase the number of observations per group and, therefore, increase the statistical power available. An alternative approach would be to increase the level of parasite infection although this would require careful titration to ensure that the infection does not overwhelm any protective immune response. Increasing the parasite dose should allow meaningful worm counts to be undertaken with the possibility of determining whether any reductions in faecal egg output resulted from reductions in parasite establishment or in fecundity effects.

A further consideration is the tissue migratory phase of the life cycle of *N. brasiliensis*. It is possible that the mechanism of protection conferred by immunisation might act during this phase of the life cycle and not during the intestinal phase. Vaccination with AChE is unlikely to be protective during this phase, as native AChE is expressed strongly in the L4 and adult stages of the worm present in the gut of the rat but not in the migratory L3 stage (Sanderson and Ogilvie, 1971). However, it would be important to confirm this before conducting any more advanced experiments. Additionally, the stage specificity of expression of any other antigen found to be protective in this system might be relevant to the stage of the life cycle in which the protection was expressed.

The effect of vaccination on different *N. brasiliensis* life cycle stages could be tested by recovering larvae from different time-points through the course of infection (Sanderson and Ogilvie, 1971). A larger challenge dose of L3 than was used in these experiments and a cull of animals through an infection would be required to perform the experiment. Any antigens found to be protective during the larval migratory phase might in any case have relevance to studies into vaccination hookworms or *D. viviparus* with which the earlier tissue migratory stages of *N. brasiliensis* bears similarities.

To summarise, this study has indicated that the *N. brasiliensis* / rat model is a useful tool for the evaluation of antigens and delivery systems in the development of recombinant vaccines against ruminant nematodes. The protection trial data suggest that recombinant worm AChE might be a useful component of a vaccine against intestinal nematodes, confirming previous work on this enzyme. The data further suggest that the mode of delivery and antigen used in trials of recombinant antigens may be important in the ability to provoke a protective immune response against GI nematodes. This finding might have important implications for the testing of recombinant vaccine candidates. Finally it is possible to speculate that this and future work in this system may play an important part in the development of recombinant vaccines against intestinal nematodes of ruminants.

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Appendix 1- Working Buffers and Solutions

Tris / Acetic acid / EDTA (TAE) buffer: 40mM Tris / acetic acid, 2mM EDTA (pH 8) in H_2O.

PAGE Separating gel buffer: 1.5 M Tris, 1.5 mM SDS in H_2O.

PAGE Stacking gel buffer: 0.25 M Tris, 1.5 mMSDS in H_2O.

PAGE Electrode buffer: 25 mM Tris, 0.2 M glycine, 3.5 mM SDS in H_2O.

PAGE Sample buffer: 0.125 M Tris pH 6.8, 20% glycerol (v/v), 1.5 M SDS, 10% ß-mercaptoethanol (v/v), 0.025% bromophenol blue.

PAGE Non-reducing sample buffer: 0.125 M Tris pH 6.8, 20% glycerol (v/v), 1.5 M SDS, 0.025% bromophenol blue.

Blotting anode buffer 1: 0.3 M Tris in H_2O.

Blotting anode buffer 2: 0.025 M Tris in H_2O.

Blotting Cathode buffer: 0.025 M Tris / glycine pH 8 in H_2O.

Tris / HCl buffer: 10mM Tris adjusted to desired pH with HCl in H_2O.

Tris buffered saline (TBS): 50 mM Tris / HCl pH 8, 150 mM NaCl in H_2O.

Tris buffered saline with Tween-20 (TBST): TBS with 0.05% Tween-20 (v/v).

Carbonate/bicarbonate buffer: 0.25 mM Na_2CO_3 adjusted to desired pH with 0.25 mM $NaHCO_3$ in H_2O.

Phosphate buffer: 0.1 M K_2HPO_3 adjusted to the desired pH with 0.1 M KH_2PO_4 in H_2O.

Phosphate buffered saline (PBS): 0.1M phosphate buffer pH 7.4, 0.9% NaCl (w/v) in H_2O.

Phosphate buffered saline with Tween-20: PBS with 0.05% Tween-20 (v/v) in H_2O.

Coomassie blue stain: 0.25% Coomassie brilliant blue, 40% methanol (v/v), 10% acetic acid (v/v) in H_2O.

Destain: 30% methanol, 10% acetic acid in H_2O.

Agarose loading buffer: 40% sucrose, 0.25% bromophenol blue in H_2O.

SOC media: 2% Trypton, 0.5% yeast extract, 0.05% NaCl, 0.1 M Mg SO_4, in H_2O.

Luria broth (LB): 1% Trypton, 0.5% yeast extract, 1% NaCl.

LB Agar: 1% Trypton, 0.5% yeast extract, 1% NaCl, 1.5% Agar.